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EFFECTS OF TOXIC ORGANICS ON PHOTO-
SYNTHETIC REOXYGENATION

Ju-Chang Huang, et al

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EFFECTS OF TOXIC ORGANICS ON
PHOTOSYNTHETIC REOXYGENATION

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this document may be better
studied on microfiche.

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Federal Water Pollution Control Administration
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III

EFFECTS OF TOXIC ORGANICS ON
PHOTOSYNTHETIC REOXYGENATION

ABSTRACT

This dissertation describes the potential reduction of dissolved oxygen in ponds and streams due to chlorophyll inhibition resulting from certain industrial wastes. Emphasis is placed on the laboratory techniques developed for this study and the changes that should be incorporated in waste stabilization pond designs.

The relative toxicity of 33 phenolic-type compounds and 8 pesticides to Chlorella pyrenoidosa was evaluated. Phenolic-type compounds selected for this study included those with mono- and poly- substituted groups such as -Br, -Cl, -CH₃, -NO₂, -NH₂, -OH. The pesticides selected included DDT, lindane, 2,4-D, sodium salt of 2,4-D, 2,4,5-T, Dow sodium TCA, Dow Esteron 99, and Dow Formula 40.

The toxicity of organic compounds was evaluated by two methods: First, algal cultures were grown in test tubes which were chemostated at 25°C and under continuous illumination for a period of 72 hours. Environmental factors were rigidly controlled. Knop's solution, including the Hutner-EDTA microelement system, was used as the culture medium. A stream of 5% carbon dioxide in air gas

mixture was supplied to provide the inorganic carbon source and also to keep the algal cells in suspension. The toxicity was evaluated by measuring changes in chlorophyll content of the algal suspensions every 24 hours. Second, the Warburg manometric technique was used to measure the photosynthetic gas exchange that occurs when a carbonate-bicarbonate buffer is used as the suspending fluid. The concentration of carbon dioxide is well stabilized. Errors of less than one percent can be expected from application of the direct method.

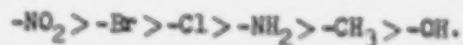
The results show that in test tube cultures the decrease in chlorophyll content is generally logarithmic to the concentration of toxic organics. In the case of phenolic-type compounds, the toxicity is a function of the substituent groups and their relative position. The manometric measurement of photosynthetic gas exchange reveals that oxygen evolution is suppressed by the toxic organic. Actual model studies involving waste stabilization ponds substantiate the predicted decrease in dissolved oxygen. Therefore, design modifications must be considered. A toxicity-compensation factor must be added to established equations which are used to design waste stabilization ponds.

CONCLUSIONS

1. The presence of toxic organic compounds will reduce chlorophyll synthesis, thereby decreasing the photosynthetic oxygenation. For most of the phenolic-type compounds and certain pesticides the decrease in chlorophyll content is generally logarithmic to the concentration of toxic organics.

2. The toxicity of phenolic-type compounds is generally a function of the substituent groups and their relative substitution position.

a.) In general, introduction of a substituent group into the phenol nucleus increases the toxicity to Chlorella pyrenoidosa. Nitrated and halogenated phenols have higher toxicities than aminated and alkylated phenols. The effectiveness of the substituent group in raising the toxicity decreases in the following order:



b.) Introduction of halogen atoms to phenol greatly increases the toxicity to Chlorella pyrenoidosa. The toxicity increases with increasing numbers of halogen atoms. Bromophenols generally have higher toxicities than the corresponding chlorophenols. Among the mono-halogenophenols, meta isomers are the most toxic, para isomers the next, and ortho isomers the least.

c.) Introduction of a methyl group to the phenol nucleus slightly increases the toxicity. The introduction of a second methyl group further increases the toxicity. The substituted position of the alkyl group does not significantly affect the

relative toxicity.

d.) Introduction of a nitro group to phenol greatly increases the toxicity to Chlorella pyrenoidosa. However, a second nitro group added to phenol decreases the toxicity as compared with the mono-nitrophenols. Among the three nitrophenols, para isomer is the most toxic, ortho isomer the next, and meta isomer the least. The addition of a methyl group to 2,4-dinitrophenol does not alter substantially the toxicity to algae.

e.) Introduction of an amino group to the phenol nucleus moderately increases the toxicity. Ortho-aminophenol has a higher toxicity than the para and meta isomers. The addition of a sulfonic group to o-aminophenol greatly decreases the toxicity to Chlorella pyrenoidosa.

f.) Introduction of a hydroxyl group to the phenol nucleus generally decreases the toxicity. An exception is hydroquinone. The relative toxicity of the three dihydric phenols is generally consistent with their strengths of reducing powers, para isomer being the highest, ortho isomer the next, and meta isomer the least. Further addition of hydroxyl group into the phenol nucleus does not increase its toxicity.

3. Lindane has a toxicity value approximately 100 times as much as phenol and is the most toxic compound among the eight pesticides investigated. The compounds of 2,4-D, sodium salt of 2,4-D and 2,4,5-T have very similar toxicity values which are about two times as much as that of phenol. DDT, Dow sodium salt of TCA, Dow Esteron 99 and Dow Formula 40 are relatively non-toxic to algae.

4. The toxicity of a combination of compounds is additive provided there is no chemical interreaction.

5. Actual model studies involving laboratory-controlled waste stabilization ponds substantiate the predicted decrease in dissolved oxygen as well as the treatment efficiency. The design standards of waste stabilization ponds can be improved in accordance with the toxicity of the waste. That is, a toxicity-compensation factor is suggested to be added to the design equation. A general design equation for a single facultative waste stabilization pond can be developed in the following form:

$$V = 1.50 \times 10^{-8} \cdot Q \cdot Y \cdot [\theta^{(T_0 - T)}] t_0 \cdot e^{K \cdot \left(\frac{C_0}{k_0 t_0 + 1} \right)}$$

where V = designed volume of the pond, acre-feet,
 Q = quantity of influent flow, gallons/day,
 Y = 5-day 20°C BOD, mg/L,
 θ = temperature coefficient, depending on the specific waste;
for example, θ being 1.085 for certain industrial wastes,
 T_0 = observed maximum operation temperature (based on the maximum
monthly temperature), °C,
 T = expected minimum operation temperature (based on the minimum
monthly temperature), °C,
 t_0 = reaction time required for a given treatment at T_0 and k_0 , days,
 e = base of the natural logarithmic system,
 K = toxicity constant (for algal oxygenation) of the waste,
 C_0 = original concentration of the waste, mg/L BOD, or mg/L dry
weight (if applicable),
 k_0 = observed rate constant of biodegradation of the waste at T_0 .

TABLE OF CONTENTS

<u>CHAPTER</u>		<u>PAGE</u>
	ACKNOWLEDGMENTS	iii
	ABSTRACT	iv
	CONCLUSIONS	vi
	TABLE OF CONTENTS	ix
	LIST OF TABLES	xi
	LIST OF FIGURES	xiii
I.	INTRODUCTION	1
	Objectives	1
	Scope	2
II.	LITERATURE REVIEW	4
	General Aspects of Toxicity Study	4
	Toxic Effects of Organics on Algae	6
	Degradation of Pure Organics by Bacteria	12
	Toxic Effects of Organics on Bacteria and Other Microorganisms	16
	Algae and Waste Stabilisation Ponds	18
III.	CHLOROPHYLL INHIBITION IN TEST-TUBE CULTURES	23
	Experimental Equipment	23
	Experimental Procedure	28
	Test Procedure	29
	Selection of Test Period	30
	Extraction of Chlorophyll	32
	Standard Curve for Chlorophyll Determination	32
	Culture Medium	33
	Algal Source	34
	Selected Organic Compounds	34
	Effect of Initial Algal Mass on the Toxicity	38
	Difference in Toxicities between Conditions of Continuous Illumination and Alternative Light and Dark	41
	Comparison between Chlorophyll Synthesis and Algal Growth	46

TABLE OF CONTENTS (Cont'd.)

<u>CHAPTER</u>		<u>PAGE</u>
	Evaluation of Results	49
	Phenol	50
	Mono-Halogenated Phenols	52
	Poly-Halogenated Phenols	59
	Alkyl-Substituted Phenols	64
	Nitro-Substituted Phenols	72
	Amino-Substituted Phenols	77
	Dihydric and Trihydric Phenols	82
	Pesticides	87
	Toxicity of the Combination of Two Organic Compounds	97
IV.	SUPPRESSION OF PHOTOSYNTHETIC GAS EXCHANGE	101
	Experimental Equipment	101
	Measurement of Photosynthesis	102
	Test Procedure	103
	Evaluation of Results	104
V.	MODEL WASTE STABILIZATION PONDS	114
	Experimental Setup of Model Waste Stabilization Ponds	114
	Synthetic Substrate	115
	Experimental Design of Influent Waste Streams and Detention Time for Model Ponds	117
	Influent Feedings and Effluent Withdrawals	119
	Experimental Analyses	120
	Evaluation of Analytical Results	121
VI.	DISCUSSION	130
	Relationship between the Toxicity and Chemical Structure of Phenolic-Type Compounds	130
	Design Modifications of Waste Stabilization Ponds	135
	Limitations of K Values	143
	APPENDICES	145
	I. CHLOROPHYLL EXTRACTION	146
	II. STANDARD CURVE FOR CHLOROPHYLL DETERMINATION	149
	III. APPARATUS AND METHOD FOR CONTINUOUS ALGAL CULTURE	151
	BIBLIOGRAPHY	158

LIST OF TABLES

<u>Table</u>	<u>Title</u>	<u>Page</u>
3 - 1	Macroelements of Knop's Solution	33
3 - 2	Hutner-EDTA Microelements for Knop's Solution	33
3 - 3	Structural Formula and Certain Physical Constants of the Selected Organic Compounds	35
3 - 4	Effect of Initial Algal Mass on the Toxicity	38
3 - 5	Chlorophyll Synthesis in the Presence of Phenol under Continuous Illumination	42
3 - 6	Chlorophyll Synthesis in the Presence of Phenol under Alternative Light and Dark	42
3 - 7	Comparison between Chlorophyll Synthesis and Algal Growth	47
3 - 8	Test Data for Bromophenols	54
3 - 9	Test Data for Chlorophenols	56
3 - 10	Test Data for Poly-Halogenated Phenols	61
3 - 11	Test Data for Cresols	66
3 - 12	Test Data for Xylenols	67
3 - 13	Test Data for Nitro-Substituted Phenols	73
3 - 14	Test Data for Amino-Substituted Phenols	78
3 - 15	Test Data for Dihydric and Trihydric Phenols	84
3 - 16	Test Data for Pesticides	89
3 - 17	Toxicity of the Combination of Two Compounds	99
4 - 1	Characteristics of the Suspending Fluid	102
4 - 2	Inhibitory Effects of Phenol and Chlorinated Phenols	105
4 - 3	Inhibitory Effects of Nitrated Phenols	106

LIST OF TABLES (Cont'd.)

<u>Table</u>	<u>Title</u>	<u>Page</u>
5 - 1	Composition of Starlac	116
5 - 2	Preparation of a Synthetic Substrate with a COD of 990 mg/L	117
5 - 3	Analytical Results of Five Ponds' Operation	122
6 - 1	Toxicity Constants of Selected Compounds	131

LIST OF FIGURES

<u>Figure</u>	<u>Title</u>	<u>Page</u>
3 - 1	Cross-Sectional View of Test Apparatus	25
3 - 2	General Pictorial View of Test Apparatus	26
3 - 3	Chlorophyll Synthesis in Algal Cells Resuspended in the Fresh Medium	31
3 - 4	Effect of Initial Algal Mass on the Toxicity	39
3 - 5	Chlorophyll Synthesis in the Presence of Phenol under Continuous Illumination	43
3 - 6	Chlorophyll Synthesis in the Presence of Phenol under Alternative Light and Dark	43
3 - 7	Difference in Toxicities between Conditions of Continuous Illumination and Alternative Light and Dark	43
3 - 8	Comparison between Chlorophyll Synthesis and Algal Growth	48
3 - 9	Effects of o-Bromophenol	55
3 - 10	Effects of m- and p-Bromophenols	55
3 - 11	Effects of o-Chlorophenol	57
3 - 12	Effects of m- and p-Chlorophenols	57
3 - 13	Toxicity Constants of the Six Mono- Halogenated Phenols	57
3 - 14	Effects of 2,4-Dichlorophenol	62
3 - 15	Effects of 2,4,5- and 2,4,6-Trichlorophenols	62
3 - 16	Effects of Pentachlorophenol	62
3 - 17	Toxicity Constant of 2,4-Dichlorophenol	63
3 - 18	Toxicity Constants of 2,4,5- and 2,4,6- Trichlorophenols	63
3 - 19	Toxicity Constant of Pentachlorophenol	63

LIST OF FIGURES (Cont'd.)

<u>Figure</u>	<u>Title</u>	<u>Page</u>
3 - 20	Effects of o- and m-Cresols	68
3 - 21	Effects of p-Cresol	68
3 - 22	Effects of 2,4- and 2,5-Xylenols	69
3 - 23	Effects of 2,6-Xylenol	69
3 - 24	Effects of 3,4- and 3,5-Xylenols	70
3 - 25	Toxicity Constants of Cresols and Xylenols	70
3 - 26	Effects of o- and m-Nitrophenols	75
3 - 27	Effects of p-Nitrophenols	75
3 - 28	Effects of 2,4-Dinitrophenol and 4,6-Dinitro-o-cresol	76
3 - 29	Toxicity Constants of Nitrated Phenols	76
3 - 30	Effects of o- and m-Aminophenols	80
3 - 31	Effects of p-Aminophenol and 2-Amino- 1-phenol-4-sulfonic Acid	80
3 - 32	Toxicity Constants of Aminophenols	81
3 - 33	Toxicity Constant of 2-Amino-1-phenol- 4-sulfonic Acid	81
3 - 34	Effects of Catechol and Hydroquinone	85
3 - 35	Effects of Resorcinal	85
3 - 36	Effects of Pyrogallol and Phloroglucinol	87
3 - 37	Toxicity Constants of Dihydric Phenols	87
3 - 38	Effects of DDT and Lindane	91
3 - 39	Toxicity Constant of Lindane	91
3 - 40	Effects of 2,4-D and Sodium Salt of 2,4-D	92

LIST OF FIGURES (Cont'd.)

<u>Figure</u>	<u>Title</u>	<u>Page</u>
3 - 41	Effects of 2,4,5-T	92
3 - 42	Toxicity Constants of 2,4-D, Sodium 2,4-D and 2,4,5-T	92
3 - 43	Effects of Dow Sodium TCA	93
3 - 44	Toxicity Constant of Dow Sodium TCA	93
3 - 45	Effects of Dow Esteron 99 and Dow Formula 40	94
3 - 46	Toxicity Constants of Dow Esteron 99 and Dow Formula 40	94
3 - 47	Toxicity of the Combination of Two Compounds	100
4 - 1	Inhibitory Effects of Phenol	107
4 - 2	Inhibitory Effects of o-Chlorophenol	107
4 - 3	Inhibitory Effects of m-Chlorophenol	108
4 - 4	Inhibitory Effects of p-Chlorophenol	108
4 - 5	Inhibitory Effects of 2,4-Dichlorophenol	109
4 - 6	Inhibitory Effects of 2,4, 6-Trichlorophenol	109
4 - 7	Inhibitory Effects of o-Nitrophenol	110
4 - 8	Inhibitory Effects of m-Nitrophenol	110
4 - 9	Inhibitory Effects of p-Nitrophenol	111
4 - 10	Inhibitory Effects of 2,4-Dinitrophenol	111
5 - 1	Temperature Profile in Model Ponds	125
5 - 2	Dissolved Oxygen Profile in Model Pond I	125
5 - 3	Dissolved Oxygen Profile in Model Pond II	126
5 - 4	Dissolved Oxygen Profile in Model Pond III	126
5 - 5	Dissolved Oxygen Profile in Model Pond IV	127
5 - 6	Dissolved Oxygen Profile in Model Pond V	127
A3 - 1	Continuous Culture Apparatus	153

CHAPTER I

INTRODUCTION

Phenolic compounds, pesticides and other toxic organics are becoming increasingly important and widespread in soil, water and industrial wastes. The presence of excessive concentrations of these toxic organics may effectively interfere with stream reaeration or decrease the effectiveness of waste stabilization ponds by destroying algae and thereby inhibiting the photosynthetic reoxygenation.

The popularity and importance of waste stabilization ponds to industry have been well-documented. Porges in 1962 (61) reported that 31 types of industrial wastes were treated by 827 waste stabilization ponds in the United States. The number of ponds being used today is increasing rapidly, but unfortunately, little is known about the effect of "shock" loading, which appears as a result of periodic slug releases of certain toxic wastes, on the natural oxygenation system. In this connection a study was undertaken to determine the relative toxicity of selected organics to algae.

Objectives

The purpose of this research was to evaluate the relative magnitude of the toxicity factor found in the modified Hermann-Gloyne design formula for waste stabilization pond. Specifically, the design objective was to establish the effect of selected organics to a culture of Chlorella pyrenoidosa.

Emphasis was placed on the laboratory techniques developed

for this study and the changes that should be incorporated in waste stabilization pond designs.

Scope

Chlorella pyrenoidosa was selected as the test organism because it is commonly found in streams and waste stabilization ponds. Also, Chlorella are most frequently used in physiological and biochemical studies.

Thirty three phenolic-type compounds and eight pesticides were selected in this study. Phenolic-type compounds selected for this study included those with mono- and poly- substituted groups such as -Br, -Cl, -CH₃, -NO₂, -NH₂, -OH. The pesticides selected included DDT, lindane, 2,4-D, sodium salt of 2,4-D, 2,4,5-T, Dow sodium TCA, Dow Esteron 99, and Dow Formula 40.

The toxicity of organic compounds was evaluated by two methods: First, algal cultures were grown in test tubes which were chemostated at approximately 25°C and under continuous illumination for a period of 72 hours. Environmental control was rigidly established. Knop's solution, including the Hutner-EDTA microelement system, was used as the culture medium. A stream of 5% carbon dioxide in air gas mixture was supplied to provide the inorganic carbon source and also to keep the algal cells in suspension. The toxicity was evaluated by measuring changes in chlorophyll content of the algal suspensions every 24 hours. Second, the Warburg manometric technique was used to measure the photosynthetic gas exchange that occurs when a carbonate-

bicarbonate buffer was used as the suspending fluid. The carbon dioxide concentration is well stabilized. Errors of less than one percent can be expected from application of the direct method.

Actual model waste stabilization ponds were used to investigate the effect of the presence of toxic compound on the pond's operation efficiency which is interpreted in terms of the existing dissolved oxygen level and the COD removal within the pond system.

CHAPTER II

LITERATURE REVIEW

Increase in complexities of the rapid expanded chemical industry and the need for water reuse necessitate greater emphasis on toxicity determinations of waste water. In recent years a great deal of interest and research has been concentrated on the treatment and disposal of industrial wastes containing toxic materials. This chapter presents an evaluation of the literature in the following areas which are related to this study: general aspects of toxicity study, toxic effects of organics on algae, degradation of pure organics by bacteria, toxic effects of organics on bacteria and other microorganisms, algae and waste stabilization ponds.

General Aspects of Toxicity Study

Up to now the methods for toxicological testing, definitions and interpretations of the results have not been standardized yet. However, a practical problem can usually be solved by a certain step depending on the individuality and originality of the problem. According to Gerarde (26) toxicology is more than the routine testing and analysis of chemicals; it is a biological research designed to find out as quickly as possible the toxic effects of a chemical on a given organism.

The statistical interpretation of the experimental data is the most important part for a toxicity study. A satisfactory evaluation can only be derived by experience and judgment coupled with sound training

in both the biological and chemical sciences. Consequently, the degree of toxicity may be related to the chemical structure, substitution group, molecular weight, or some other chemical parameter.

In interpreting the toxicity of a chemical with respect to a given organism, the environmental factors must be well defined. For example, Fitzgerald, Gerloff and Skoog (22) showed that 2,3-dichloro-naphthoquinone is an inhibitor to blue-green algae at concentrations from 2 to 10 $\mu\text{g/L}$. However, under alkaline conditions this chemical is hydrolyzed to 2-chloro-3-hydroxynaphthoquinone, which is not an effective algicide at concentrations of 1 to 2 mg/L .

Fitzgerald and Faust (21) have defined the algicidal and algistatic properties of a chemical. Whether a toxic level of a chemical is algicidal or algistatic is determined by removing a sample of algal suspension from the environment of the toxic chemical and placing it in a suitable culture medium. If the subculture of the algae does not revive, the concentration of the chemical is considered as algicidal. If the algae in the subculture start to grow under appropriate incubation, the concentration is said to be algistatic.

Experts on the subject of toxicology are divided in their opinions regarding the evaluation of toxicity. Some of them prefer to evaluate by tests on living organisms and others prefer to measure specific toxic substance by chemical analysis. In general, toxicity of organic chemicals to aquatic life is most often evaluated in the laboratory by aquarium bio-assay using fish as the test organism. The result is normally expressed as the median tolerance limit (TL_{50}), defined

as the concentration at which 50 percent of the test fish survived, for a certain test period. The vital factors which must be clearly described are selection of test organism with respect to species, acclimatization and physical fitness of these specimens, number of specimens, duration of test, feeding habits during the test, sources of water supply and environmental factors such as temperature, pH, liquid depth and aeration etc.

Toxic Effects of Organics on Algae

Green, McCarthy and King (30) first in 1939 investigated the inhibition of respiration and photosynthesis in Chlorella pyrenoidosa by the organics, such as thiourea and 8-hydroxyquinoline, which inhibit copper catalysis. The results showed that typical copper "poison" will inhibit both photosynthesis and respiration in this alga, photosynthesis being more sensitive than respiration.

Korsep (39) conducted a laboratory study of toxicity of 29 various organic compounds and one petrochemical waste to a species of green alga, Chlorella pyrenoidosa. The data indicated that organic acids are least toxic to this alga, followed by alcohols and amines, alkylhalides, and ketones. Compounds of the benzene series exhibited the highest toxicity.

Thirumurthi and Gloyna (72) also recently evaluated the relative toxicity of 60 selected organics to Chlorella pyrenoidosa. The chemicals selected included lower chain fatty acids, dicarboxylic acids, alcohols, aldehydes and some other organics. The results showed that the straight chain fatty acids with odd number of carbon atoms are more toxic than the corresponding acids with even number of carbon

atoms; also, the straight chain fatty acids with four and five carbon atoms are more toxic than the corresponding branched chain acids. The toxicity of six dicarboxylic acids from C_2 to C_7 decreases with increasing number of carbon atoms per molecule for the first three acids in the chain, however, the toxicity increases with increasing number of carbon atoms for the fifth, sixth, and seventh members. General observations seem to indicate that some of the straight chain organic compounds exhibit a greater degree of toxicity than the compounds involving branched chains.

Espino (15) studied the toxicity of some organic acids and alcohols to Chlorella pyrenoidosa by using the combined culture with bacteria. The data reflected that the toxicity of organics to algae is not materially affected by the presence of bacteria. Ukeles (74) tested 17 different toxicants, primarily synthetic pesticides, against the growth of five species of algae which included Monochrysis lutheri, Dunaliella euchlora, Chlorella sp., Protococcus sp., and Phaeodactylum tricornutum. The chemicals tested included organic phosphates, phenol, chlorinated hydrocarbons and substituted urea compounds.

Lefrancois and Quellet (43) conducted experiments on the photosynthetic assimilation of labeled carbon dioxide by Scenedesmus in the presence of ethanol and o-phenanthroline and disclosed that the fixation of labeled carbon dioxide into some key compounds such as phosphoglyceric acid is blocked, while the rate of fixation into other compounds such as glycolic acid and the simple amino acids remains nearly normal or is accelerated. It was also shown that the inhibiting power of the straight chain alcohols increases correspondingly with the

number of carbon atoms in the molecule.

Asada and Kasai (5) found that certain bisulfite addition compounds of aldehydes and ketones, known as α -hydroxysulfonates, inhibit the photosynthetic carbon dioxide fixation on Chlorella cells. Bisulfite addition compounds of glyoxal, glyoxylate and benzaldehyde are more effective in their toxicity than those of formaldehyde and acetaldehyde.

Fitzgerald, Gerloff and Skoog (22) investigated the toxicity of 300 chemicals, particularly quinone derivatives, to a culture of blue-green algae and found that the general relationship between toxicity and chemical structure of the quinone-type compounds are consistent with the following statements made by Fieser (16) as regard to the reactivity of the hydrogens of the compounds: a.) Any compound with two H atoms which can be simultaneously removed by oxidization from -OH or -NH₂ groups which are ortho or para to each other, or adjacent to one another, or which bear some similar relationship, will give rise to a fairly stable oxidant which, in general, will exhibit toxic properties. b.) The structure of the rings and the additions to the rings merely increase or decrease the effects of the redox character and thus the toxicity. Ortho and para directing groups, such as -Cl, -CH₃, -OH, -OCH₃, -NH₂, -NHCOCH₃, -N(CH₃)₂ and -CH₂COOH, usually weaken the bond holding the H atom to the O or N and thus tend to increase the toxicity. Meta directing groups, such as -SO₃H, -NO₂, -COOH, -COOC₂H₅, -CHO, -C≡N, -COR, -NH₃⁺ and -NR₃⁺, strengthen the hydrogen bonding to O and N and thus tend to decrease the toxicity. In view of this, 2,3-dichloronaphthoquinone and

phenanthraquinone exhibit extremely highly toxic effects on blue-green algae even at a low concentration of 2 and 80 $\mu\text{g/L}$, respectively. The algicidal concentration of chemicals will change depending upon the environmental conditions.

Maloney and Palmer (47) surveyed the toxicity of six chemicals to 30 cultures of algae. The chemicals selected were copper sulfate, 2,3-dichloronaphthoquinone (2,3-DNQ), dodecylacetamido dimethyl benzyl ammonium chloride (DAC), rosin amine D acetate (RADA), zinc dimethyl dithiocarbamate (ZDD) and rosin amine D sulfate (RADS). At 0.5 mg/L 2,3-DNQ destroyed 28 percent of the blue-green algae without being toxic to any of the diatoms and green algae. The toxicity rating was found to be: ZDD > RADA > DAC > RADS > copper sulfate > 2,3-DNQ.

Wilson and Choudhri (78) found that Chlorella and two other algae failed to survive the small application of benzene hexachloride. Lefevre and Nisbet (42) reported that secreted substances by a certain species of algae may inhibit the growth of other species. Examples of such inhibiting relations were found that Phormidium uncinatum produces a substance called phormidine which prevents the growth of Scenedesmus quadricauda; on the other hand, the latter produces scenedesmine, which inhibits Pediastrum boryanum. Safferman and Morris (65) also evaluated the algicidal properties of certain natural products and showed that the extracellular materials of 505 actinomycete and fungal isolates constitute a wide distribution of agents having algicidal activities.

Beneden (6) studied the effects of detergents on microorganisms and found that algal growth is inhibited by a quaternary ammonium base at concentrations from 1 to 100 mg/L, anionic surfactants from 10 to 120 mg/L, and arylpolyethylene glycol at concentrations over 500 mg/L.

Kessler (38) found that 2×10^{-4} M of 2,4-dinitrophenol at a pH of 4.0 completely inhibits the reduction of nitrite in Chlorella ellipsoidea; however, the reduction of nitrate to nitrite that occurs under anaerobiosis is not affected. Michiels and Dustin (53) showed that some herbicides such as 2,4-D, 3-(p-chlorophenyl)-1,1-dimethyl-urea (CMU), TCA, DNOC/ NH_4 are favorable for destruction of algae. Jeney and Zsolnai (36) found that fluoroacetic acid at a concentration of 0.00095 M inhibits the growth of two species of algae. Rai, Tehle and Kamara (63) reported that Cu, Ni, and Co complexes of benzimidazole are effective at the concentrations of 0.5 to 25 mg/L against green algae, such as Scenedesmus and Chlamydomonas, which form slimes in water circulating systems. These benzimidazole-metal complex algicides will control algae in water without the undesirable corrosion and Cu deposition on metals which are characteristic of CuSO_4 algicides.

It was reported (76) that some alkylamino-1,4-benzoquinones at the concentration of 10 mg/L are effective to kill 57 to 98 percent of an assorted algal culture, primarily Scenedesmus. The compounds included 2-dimethylamino-, 2,5-bis(dimethylamino)-, 2,5-bis(monoethylamino)-, 2,5-bis(diethylamino)-, 2,5-bis(dipropylamino)-, and 2,5-bis(dimethylamino)-3-methyl-, 2,5-bis(monoisopropylamino)-1,4-benzoquinone.

Bogdanova (9) also studied the toxicity of the following chemicals to blue-green algae including Microcystis, Aphanizomenon and Anabaena: aspartic acid, urea, streptomycin, streptocide, sulfodimezine, norsulfazole, synthomycin, salicylic acid, 4',5-dibromosalicylanilide, 2',5-dichlorosalicylanilide, N-(dichloroiodo)-4'-bromosalicylanilide, N-(dichloroiodo)-4'-chlorosalicylanilide, simazine, atrazine, 2,4-D, dikotex and DNOK. The results showed that Anabaena has the lowest resistance while Microcystis has the highest to the chemicals added.

Fitzgerald and Faust (21) evaluated the algicidal and algistatic effects of the selected chemicals to Chlorella pyrenoidosa and disclosed that algimycin 200, algimycin MT-4, algimaster, and armazide at the concentration of 3 mg/L, exalgae L.C. at 5 mg/L, steramine at 20 mg/L are algicidal in contact periods of 10 minutes to 24 hours. Fitzgerald (20) has recently presented a review of some of the factors affecting the laboratory testing and practical applications of chemicals toxic to algae.

It was reported (1) that dodecyl guanidine hydrochloride and trimethylalkyl ammonium chloride at the concentration of 2 mg/L are algicidal to all the blue-green algae and diatoms tested and are algistatic to all but two species of green algae. The compound 1,3,4,6-tetrachloroglycouril at the same concentration is algicidal to all species of green algae and diatoms tested.

Izawa (35) studied the inhibition of photosynthesis in Rhodopseudomonas palustris by the reduced forms of dyes such as methylene blue, brilliant cresyl blue and toluidine blue and concluded

that these dyes might act in two possible ways and thus inhibit the photosynthetic metabolism. These dyes may act as oxidation-reduction catalysts and form a short circuit in the photosynthetic process; thus the flow of electrons for the reduction of carbon dioxide is blocked. Another possibility is that the reduced forms of these dyes may affect photosynthesis by merely reducing some essential components in the metabolism.

Antonides and Tanner (2) tested the algicidal properties of armazide, a swimming pool additive, and found that this chemical is effective in controlling the algal growth at a concentration of 4.5 mg/L. It was suggested that the hardness encountered in the natural waters of different geographic areas should be considered while determining the dose of armazide in swimming pools.

Joliot (37) has recently examined the kinetics of photosynthetic oxygen emission in Chlorella pyrenoidosa in the presence of chloromethylurea and hydroxylamine. The results showed that the function $V=f(E)$, where V is the stationary speed of oxygen emission and E equals the concentration of the photochemical compound of system II, is convex in the presence of chloromethylurea and strictly linear in the presence of hydroxylamine.

Degradation of Pure Organics by Bacteria

In recent years there has been an increasing interest in the metabolism of pure organic compounds by sewage organisms. It is believed by many investigators that such information will contribute valuable knowledge to the understanding of the development of heterogeneous populations and in evaluating the treatability of toxic industrial wastes.

One of the first investigations employed a pure organic compound was conducted by Ruchhoft and his co-workers in 1940 (64). Since then there have been a number of studies conducted by Gellman and Heukelekian (24, 25), Mills and Stack (54), Placak and Ruchhoft (60), McKinney and Jeris (51, 52) etc. In general, the results showed that a period of acclimation or adaptation is necessary for optimum efficiency of biological system.

From research on the utilization of various organic substrates by activated sludge, Placak and Ruchhoft (60) concluded that the rate of biochemical change is a function of the individual compound rather than of the class to which the compound belongs. Later Mills and Stack (54), using acclimated non-flocculent aerobic cultures, found that biological oxidation of pure organic compounds is related to the chain structure, molecular size, and the functional groups present in the molecule. The significance of chemical structure as related to acclimation and metabolism had also been observed by other investigators (25, 51).

Heukelekian and Rand (33) presented biochemical oxidation data for many organic chemicals and assembled various compounds into fourteen broad groups on the basis of certain chemical similarities. Most of the data were for a 5-day incubation period and standard dilution method was commonly employed, using sewage as seed. There were also a number of results using manometric methods and specially adapted seeds and cultures. Ludzack and Ettinger (45) also presented valuable information relating the various chemical structures to biological assimilation.

In investigating the bio-oxidation of low molecular weight alcohols by activated sludge, McKinney and Jeris (51) concluded that normal alcohols of higher molecular weight than methanol undergo oxidation, through formation of aldehydes, and organic acids to carbon dioxide and water. Some of the iso-alcohols undergo similar oxidation as the normal alcohols and some are retarded. The 1 and 3 carbon alcohols are less readily oxidized than the 2 and 4 carbon alcohols. Sludges acclimatized to alcohols can oxidize structurally related compounds.

Formaldehyde (24) can be oxidized by a pink flocculent growth of Bacterium methylicum. The sludge resulting from this growth settles rapidly. Ninety-five percent BOD reduction is attained with concentrations up to 1750 mg/L of formaldehyde in 24 hours of aeration. The maximum observed oxidation rate was 190 mg/L/hr. The minimum BOD to N ratio was 40 : 1. A minimum operating pH of 6.0 was reported. Dickerson (10) also reported an efficient destruction of formaldehyde by a system consisting of a trickling filter with a 40 to 1 recirculation followed by a lagoon. Nelson (58) found that 99 percent BOD reduction can be attained from the oxidation of oxalic acid at feed levels up to 2000 mg/L by activated sludge at an operating pH range of 2.3 to 3.5.

McKinney (50) found that sludge acclimatized to phenol can oxidize o-cresol, m-cresol, p-cresol, benzoic acid and benzaldehyde in concentrations up to 500 mg/L. Placak and Ruchhoft (60) reported that o- and p-chlorophenol showed high resistance to bacterial destruction, tyrosine, formaldehyde, oxalic acid and compounds containing -SH and -CN groups were resistant to sludge treatment. Furfural was slower

to respond to biological oxidation and was lost from substrates by vaporization especially under aeration. Placak and Ruchhoft also reported that many carbohydrates, alcohols, and amino acids could be biologically oxidized or converted to cell substance at rapid rates by activated sludges.

Tabak, Chambers and Kabler (71) recently investigated the decomposition of phenolic compounds and aromatic hydrocarbons by phenol-adapted bacteria. Among 104 compounds tested, significant oxidative activity occurred with 65. In general, dihydric phenols were oxidized while trihydric phenols were not. Cresols and dimethylphenols were oxidized while chloro-substituted phenols were biologically resistant. Benzoic and hydroxybenzoic acids were oxidized while sulfonated, methoxylated, nitro, and chlorobenzoic acids were not; m-toluic acid was utilized but not the o- and p-isomers. Benzaldehyde and p-hydroxybenzaldehyde were oxidized. In general, nitro- and chloro-substituted compounds and the benzene were difficult to oxidize.

Bogan and Sawyer (8) reported that, within variable limits, all the principal anionic and non-ionic detergents are susceptible to some degree of bio-oxidation. The alkylsulfates, sulfonated fatty acid amides, esters and derivatives of low molecular weight polyethylene glycols undergo rapid oxidation while the alkylaryl sulfonates, alkylphenoxy polyglycols and derivatives of high molecular weight polyethylene glycols undergo slow oxidation. The theory offered is that only the smaller ether molecules can diffuse through cell membranes and reach the active enzyme system.

Toxic Effects of Organics on Bacteria and Other Microorganisms

Many substances exert certain toxic effects on bacteria and other microorganisms. As a consequence, partial or complete inhibition of biological oxidation process may result, depending on the toxic substance and concentration present. Inhibition may result from interference with the osmotic balance or with the enzyme system. The concentration of a substance which will exert a toxic effect is also influenced by other factors such as nutrient concentration, pH, temperature, and the nature of the organism. In some cases the biological population will acclimatize to a specific concentration level of a toxic substance. This acclimatization may result from a neutralization of the toxic material by the biological activity of the microorganisms or a selective growth of the culture in which only those organisms capable of metabolism in the presence of the toxic substance will proliferate.

Huang (34) investigated the toxicity of seven lower chain fatty acids and some aromatic compounds on sewage bacterial systems. The results showed that most of the selected organic compounds exhibits an inhibitory effect to sewage organisms at concentrations above 1000 mg/L. However, the addition of a degradable compound to a test culture containing a selected compound tends to reduce the initial toxicity of this organic compound. For the seven lower chain fatty acids tested, the more carbon atoms contained in a compound the greater the toxicity is. Phenol exhibits some toxic effect and inhibits the biological activity at concentrations above 500 mg/L.

McKinney (52) also found that phenol can serve as a bacterial food without serious toxic effects at levels up to 500 mg/L. However, Mills and Stack (54) reported that biological oxidation is inhibited during BOD incubation if the concentration of phenol is higher than 200 mg/L.

Ideas pertaining to the use of microorganisms and microcrustaceans for detecting toxic materials in water have recently been presented by Willingham and Anderson (77). For example, Bacillus subtilis is found to be sensitive to several fungicides which completely inhibit the growth of the organism. Micrococcus pyrogenes and Sarcina lutea are also shown to be sensitive to many pesticides including DDT, chlordan, aldrin, dieldrin and several others. The levels of detection were reported approximately 1.0 mg/L or less. Furthermore, there is a group of organisms, including bacteria, fungi, algae and insects, which have the ability to produce light. Therefore, it was also suggested that bioluminescent system could be used for the detection of toxic substances in the polluted waters (77). Examples of these organisms are: bacteria, Achromobacter fischeri; fungi, Armillaria mellea; algae, Noctiluca sp.; insects, Photinus pyralis (fire-fly).

The anti-bacterial activity of a series of quaternaries prepared from hexa methylene tetramine (HMT) and halo hydrocarbons was discussed by Scott and Wolf (66). The compounds having a nitrogen bonded to a carbon atom in the α -position were found to have lower toxicity.

Gershon and Parmagiani (27) evaluated the anti-bacterial activity of ring poly-chlorinated pyrimidines and found that the

addition of a side chain to the ring generally reduces the anti-microbial activity and the reduction in activity is proportional to the size of the side chain. Also, the anti-microbial activities of 8-quinolinol, its salts with salicylic acid and 3-hydroxy-2-naphthoic acid, and the respective copper chelates in liquid was studied. The results showed that in liquid culture, the salts of 8-quinolinol are not so active against bacteria as 8-quinolinol alone.

Algae and Waste Stabilization Ponds

Waste stabilization ponds are low-cost wastewater treatment devices which depend on natural aquatic processes involving bacterial and algal systems. These ponds have been referred to by many other names, including such common terms as oxidation ponds, sewage lagoons, facultative lagoons, anaerobic lagoons, redox ponds and maturation ponds, etc. A pond system may be designed to receive untreated sewage or industrial wastes, primary or secondary treatment plant effluents or excessive activated sludges, etc., depending on the treatment objectives.

Waste stabilization ponds may be used to satisfy interim waste treatment requirements, for treatment of small volumes of wastewater in isolated places, or for stabilization of large volumes of wastes. This type of waste treatment process is most suitable for locations where land is inexpensive, climatic conditions are suitable, where organic loadings may fluctuate considerably, and where funds are limited. It is estimated that this type of treatment constitutes more than 10 percent of the secondary type wastewater treatment plants in the United States (19).

A review of the literature suggests that the waste stabilization pond concept has undergone sufficient study and development to be classified as one of the major types of wastewater treatment systems. However, still little is known about the microbiology and biochemistry of the pond system. The stabilization pond is biologically more sophisticated than some other conventional treatment processes because it depends on the effective use of bacteria for degradation of putrescible organic materials and the efficient use of algae for reoxygenation purposes. The terms "symbiosis" and "commensalism" have been used to describe the mutual relationship that exists between algae and bacteria.

The biodynamic equilibrium of biological populations in waste stabilization ponds is an essential factor for an efficient treatment of wastewater. Bacteria decompose organic matter converting these complex materials into simpler compounds. The products of bacterial metabolism serve as nutrients for algae. In the photosynthesis process the algae produce oxygen which is utilized by aerobic organisms. The microbial population is kept in check by protozoa which prey on the bacteria. Invertebrates feed on algae and protozoa, and serve as a food supply for larger invertebrates or fish. The introduction into the pond system of any toxic substance which may inhibit either the microbial metabolic activities or deplete a certain microbial population will upset the biodynamic equilibrium and thus reduce the usefulness of the pond or cause the failure of the pond operation.

Algae have the capacity for both autotrophic and heterotrophic growth. Most species, under appropriate conditions, perform as facultative chemo-organotrophs and in this capacity use sugars or

organic acids for sources both of energy and of reduced carbon. These chemo-organotrophs obtain energy from reduced carbon like most bacteria. However, in stabilization ponds the primary concern is usually with the photosynthetic algae requiring only water, inorganic nutrients, and carbon dioxide. Algae utilizing the simpler nutrients are classified as autotrophic-photolithotrophs. Those growing in rich organic substrates are classified as heterotrophs. In the latter case, the algae may require growth factors to sustain photosynthesis.

Typical of the green algae in waste stabilization ponds are Chlorella, Chlamydomonas, and Euglena (29). Blue-green algae common to waste stabilization ponds are Oscillatoria, Anabaena, Phormidium, and Anacystis. In the operation of a pond Chlamydomonas and Euglena are generally the first planktonic genera to appear. Blue-green algal mats frequently develop in ponds during the summer and usually rise to the surface. Problems will also develop when detached patches of benthic algae, such as Phormidium, begin to accumulate. Euglena show a high degree of adaptability to various pond conditions and are present during all seasons and under most climatological conditions. Probably next in adaptability are Chlamydomonas, Microcystium, Ankistrodesmus, Scenedesmus, and Chlorella. Frequently Euglena and Chlamydomonas tend to dominate during the cooler weather, while the various Chlorococcales are most numerous during summer months. This latter grouping is an order under the division Chlorophyta containing Chlorella, Ankistrodesmus, Scenedesmus, etc.

Oswald, Gotaas, Ludwig and associates (39) have conducted much research in stabilization ponds and related areas. Suwannakarn (70)

investigated the temperature effects on the waste stabilization treatment. Ling (44) also conducted studies of aerated ponds and reinforced the importance of temperature in stabilization ponds.

Hermann and Gloyna (32) developed a design formulation and performance criteria for the facultative ponds. Arceivala (3) used well-known principles to develop a set of design equations which are based on the establishment of a balance between the stored energy in algal cells and the calories required to sustain the required photosynthetic activity as presented by algal growth. Marais (48) discussed the new factors in the design, operation and performance of waste stabilization ponds and confirmed that a series of ponds is more efficient than a single pond of equivalent volume. Gloyna (28) recently summarized and evaluated the pertinent experiences and research findings on the subject of waste stabilization ponds.

The importance of stabilization ponds for the treatment of industrial wastes has only recently attracted the attention of industry and regulatory agencies. Porges in 1962 (61) reported that 31 types of industrial wastes were treated by 827 waste stabilization ponds. Hart (31) advocated the use of stabilization ponds for the treatment of cyanide waste, Sorensen (67) for the treatment of wastes containing phenols, and Eichenlaub and Cox (13) for the treatment of electroplating wastes. Eckenfelder and O'Connor (12) reported that 80 percent BOD removal was obtained in the treatment of cannery and paper mill wastes by stabilization ponds. It has also been reported that the aerated ponds afforded an successful and economical method of treating many other industrial wastes (44, 7). Steel and Gloyna (69) discussed

radioactivity uptake by algae in oxidation ponds. The Limnological Department of the Academy of Natural Sciences in Philadelphia and other laboratories throughout the United States are working on solving the industrial wastes problem through the use of algal and bacterial cultures (31). Laboratory results indicate that even the most difficult wastes can be treated. However, Thirumurthi and Gloyna (73) have recently suggested that a toxicity factor must be incorporated into established design equations if there is substantial concentration of toxic material present in the wastewater. Much work remains in this area.

CHAPTER III

CHLOROPHYLL INHIBITION IN TEST-TUBE CULTURES

There were three phases of experimental studies involved in this research program: chlorophyll inhibition in test-tube cultures, suppression of photosynthetic gas exchange, and model waste stabilisation ponds. This chapter and the following Chapters IV and V will be devoted to describing each phase of study, including experimental equipment, procedure, and evaluation of results.

Experimental Equipment

The development of this experimental study required a special toxicity test device to hold the test tubes, in which algal cultures were kept, under a rigid environmental control. A generally accepted chlorophyll extraction procedure was employed.

The special toxicity test device consisted of an aquarium complete with a light source, a gas manifold and an apparatus for holding the test tubes. The aquarium was constructed of lucite and was 48 inches long, 12 inches wide, and 6.75 inches deep. The thickness of the walls and bottom, respectively, were 0.25 and 0.5 inches. To hold the test tubes which were used as the actual test vessels, two masonite boards with matched and equally spaced holes were positioned near the top and the bottom of the aquarium. Tap water with a constant head was continuously circulated through the aquarium to maintain a relatively constant temperature within $25 \pm 1^{\circ}\text{C}$. A cross-sectional view and a general pictorial view of

this test apparatus are shown, respectively, in Figs. 3-1 and 3-2.

Light source was provided from four 200-watt fluorescent lamps with attached aluminum reflectors. Each lamp was 48 inches in length. Two lamps were placed, being 0.5 inch away from the walls, on each side of the aquarium. A light-meter survey indicated that this arrangement of light source would give an intensity of 550 to 650 foot-candles at the locations where the test tubes were seated. The average light intensity was approximately 600 foot-candles.

The gas manifold was constructed in such a manner that the 9% carbon dioxide in air gas mixture could be directed to any one of the 60 outlets. A master regulator valve was used to control the tank pressure to a proper level. Attached to the central manifold were the 60 outlets. The flow through each outlet could be controlled with a needle valve.

Flexible rubber tubings were used to convey the gas mixture from the needle valves into the test tubes. Capillary glass tubes were connected to each rubber tubing and were of such length as to extend to the bottom of the test tubes. Near the upper end of each capillary glass tube was provided with a cotton plug which was inserted on the top opening of each test tube to provide for aeration and also prevent gross air-borne bacterial contaminations.

The 9% carbon dioxide in air gas mixture was used to provide an adequate carbon dioxide diffusion gradient. The flow of gas through each capillary glass tube was controlled at approximately 0.4 cc per second. This tube extended to the bottom of the test tube. Therefore, the algal cells were kept agitated and uniformly suspended.

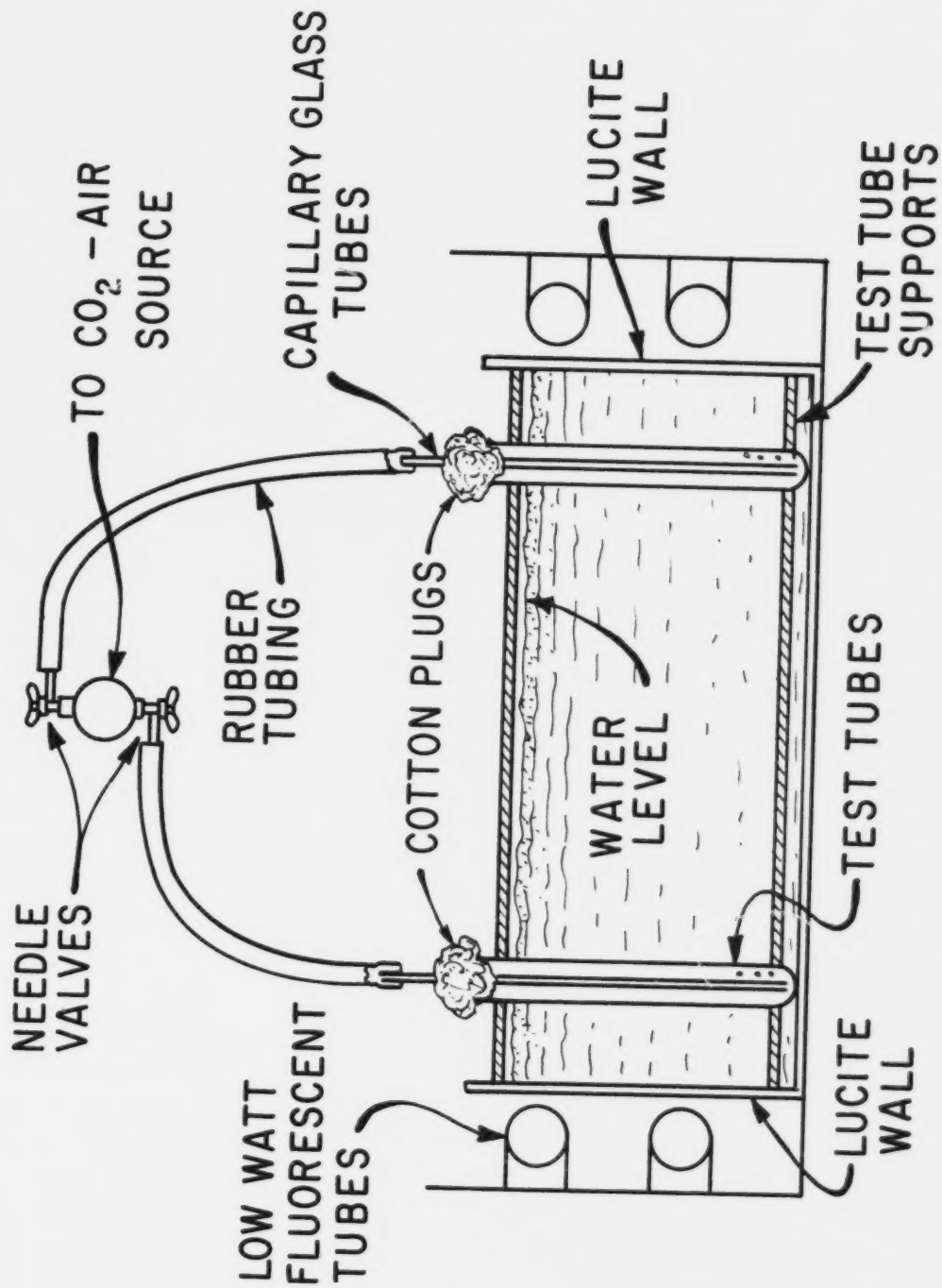


FIG. 3-1. CROSS-SECTIONAL VIEW OF TEST APPARATUS

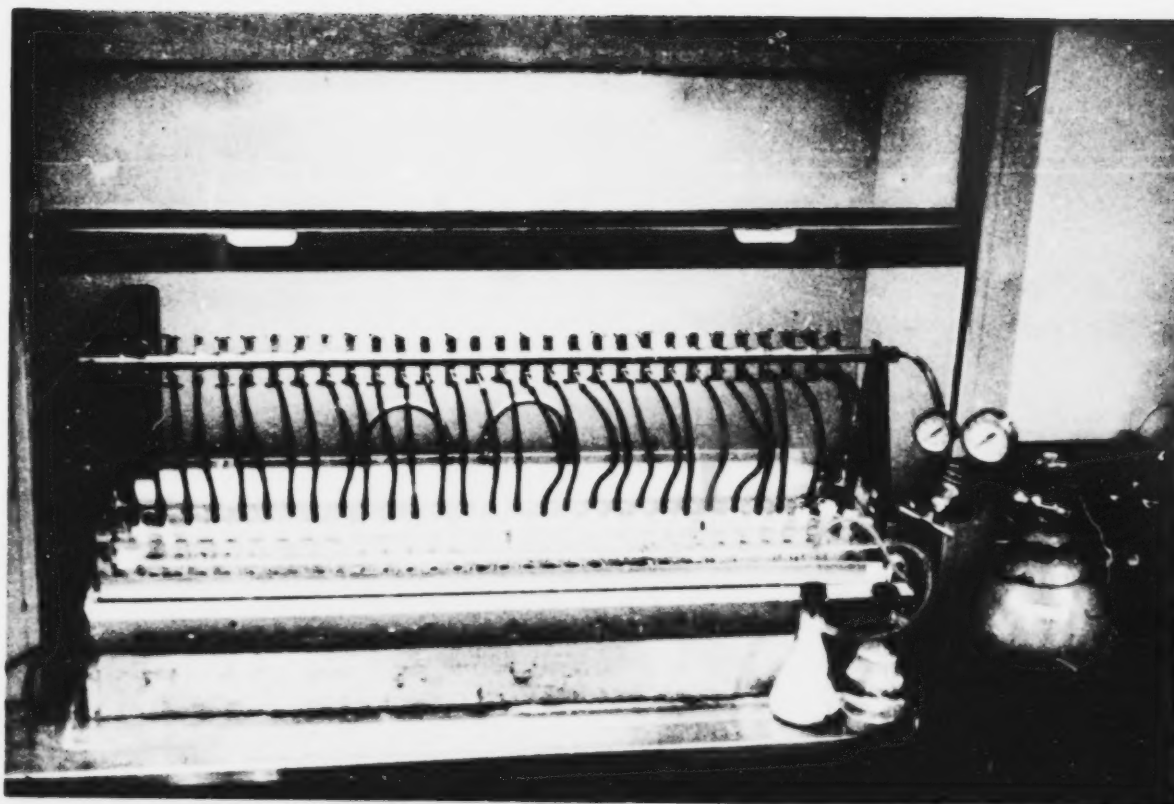


FIG. 3-2. GENERAL PICTORIAL
VIEW OF TEST APPARATUS

A Beckman Spectrophotometer, Model DB, was used for all chlorophyll analysis. This spectrophotometer is a double beam instrument for making transmittance and absorbance measurements in the 205 to 770 $m\mu$ wavelength range. Double beam operation is achieved through the use of a unique vibrating mirror assembly. This mirror system alternately directs the monochromatic beam through the sample and reference paths, providing double beam operation without using any non-common mirror in the optical path. The double beam system permits differential analysis, in which the sample is compared with a suitable reference or standard. This technique eliminates the added time factor involved in manual recording of individual. The spectrophotometer was checked with mercury lamp spectrum for the accurate wavelength prior to use.

The pH measurements were made with a Corning Model 12 Research pH Meter. This instrument is capable of performing as a pH meter covering the range pH 0 to 14, or as a voltmeter covering the ranges 0 to -1400 mv and 0 to +1400 mv. This instrument has an expand scale which allows a precise pH measurement up to 0.001 unit, and has also a temperature compensator which is calibrated in 1°C divisions for 0 to 50°C . Therefore, the effect of temperature on the pH measurement can be eliminated. Prior to use the instrument was standardized with knowing buffer solutions and the reproducibility was also checked.

Gravimetric measurements of the chemicals were made with a Sargent automatic single-pan balance, Mettler. The readability of this balance is up to 10^{-5} gram.

An Internal Clinical Centrifuge was used during the chlorophyll extraction procedures. Maximum speed attainable is 3400 rpm.

This maximum speed provides a centrifugal force of 1550 g. At this maximum speed the algal cells were completely separated from the culture medium within 15 minutes.

A Brahmall-Deane autoclave was used for sterilization of the test tubes, glassware, rubber tubings and capillary glass tubes with cotton plugs prior to each test. The autoclave was operated at 250°F with a pressure of 15 psi for 15 minutes.

Kimble Product of plain culture tubes, Series 45060, was used as the test tube. These test tubes have the size of 19 x 150 mm with a volume of 25 ml.

Experimental Procedure

The experimental procedure involved screening tests, final toxicity tests for each of the selected compounds. Screening tests were first used to find out a general threshold toxic concentration as well as a completely algicidal one. The final tests were then used to evaluate the relative magnitude of toxicity in the intermediate levels between these two extreme concentrations. The effect of the initial algal mass on the toxicity of a compound was investigated. Difference in toxicities between conditions of continuous illumination and alternative light and dark was also evaluated. Comparison between the chlorophyll synthesis and the algal growth in a control algal culture was analyzed for a period of five days. A study was also undertaken to determine the relative magnitude of toxicity of the combination of two organic compounds in an algal suspension.

Test Procedure

During the screening tests, about five different concentration levels of the tested compound were varied at wide ranges. The actual range depended on the specific chemical. These screening tests were very helpful in establishing toxicity ranges which were then used for the final tests. Also, about five intermediate concentration levels were used for the final tests. For each concentration level, three test tubes were used during the screening tests while five tubes were used during the final tests.

A typical test tube, in both screening and final toxicity tests, contained 15 ml of inorganic culture medium with a predetermined amount of the tested organic compound and 5 ml of algal source culture suspension, making a total volume of 20 ml. Knop's solution, including the Hutner-EDTA microelement system, was used as the culture medium (57). This culture medium was adjusted to a pH of 7.0 with KOH before use. The preparation and the chemical composition of this culture medium are described on page 33.

Control tubes which contained no organic chemicals in the culture medium were also used in each test. Prior to each test, test tubes covered with cap assembly, capillary glass tubes, cotton plugs, rubber tubings, glassware, and pipette were autoclaved 15 minutes for sterilization. Upon cooling, 15 ml of culture medium with a predetermined amount of the test material and 5 ml of algal source suspension were added aseptically to each test tube. Finally, this test tube assembly was attached to the gas manifold. Although, to avoid alteration of the tested organic compound, the culture media were not sterilized, the high concentration as well as the high

biological resistance of the test chemical presumably kept all experimental cultures essentially free of bacterial contaminants.

Each test was conducted over a period of 72 hours under continuous illumination. Each 24 hours, the test tubes of each organic concentration group were visually examined and compared. If there was any tube in which the algal density showed materially different from the rest of the same organic concentration group, that particular tube was eliminated from the test. Daily one of the test tubes from each organic concentration level was taken for chlorophyll analysis. The toxic effect of the organic contaminant on the algae was determined by measuring changes in the chlorophyll content.

Selection of Test Period

The test period of 72 hours was chosen mainly because the inorganic nutrients in the culture medium could only support the logarithmic increase of algae for a period of 48 hours.

If the algal suspension of a control tube was concentrated by centrifugation at the end of a 48-hour growth period and then resuspended in a fresh culture medium, the logarithmic increase of chlorophyll content in the algal suspension could be extended to the end of the third day as demonstrated in Fig. 3-3. It was recognized that when Chlorella cells are grown in a nitrogen deficient (23) or an iron deficient (62) environment, the chlorophyll synthesis in the algal cells will be markedly reduced. Therefore, any test period longer than 72 hours was considered impractical.

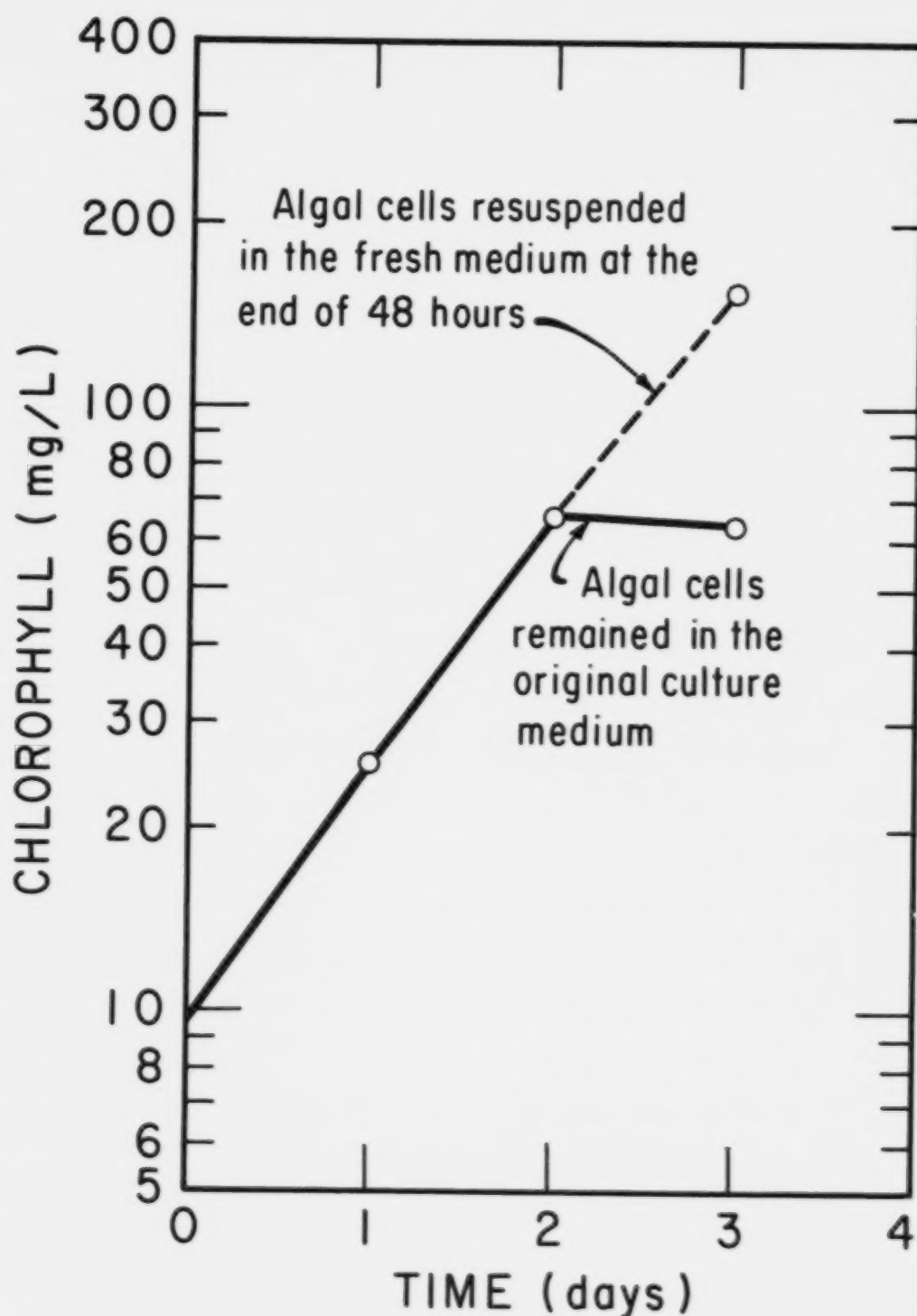


**FIG. 3-3. CHLOROPHYLL SYNTHESIS
IN ALGAL CELLS RESUSPENDED IN
THE FRESH MEDIUM**

Extraction of Chlorophyll

The chlorophyll pigment was extracted with hot methanol from a 2.5 ml algal suspension which was pipetted out of the test tube. The algal cells were centrifuged out of the culture medium, washed with distilled water and then centrifuged again. The supernatant was discarded and the remaining packed cell material was coagulated in a boiling water bath for a period of about 30 to 45 seconds depending on the amount of cells. Thereafter, the chlorophyll was extracted twice by using hot methanol.

The extracted chlorophyll solution was then dissolved in 80 percent by volume acetone-water mixture to make a final total volume of 10 ml. The chlorophyll content was then analyzed under the spectrophotometer. The detail procedure of the chlorophyll extraction method is described in Appendix I.

Standard Curve for Chlorophyll Determination

There are only two kinds of chlorophylls, a and b, present in cells of Chlorella pyrenoidosa. Content of all extracted chlorophylls which were dissolved in 10 ml of 80 percent acetone-water mixture could be determined by measuring the optical density (O.D.) at a wavelength of 652 mμ. This wavelength was optimum because various proportions of chlorophylls a and b would least affect the result (4, 46).

A standard curve was prepared to establish the relationship between the optical density and the chlorophyll content of algal cells. A detail description of the establishment of this standard curve is included in Appendix II.

Culture Medium

Knop's solution, including the Hutner-EDTA microelement system, is one of the conventional nutrients for Chlorella pyrenoidosa and was used as the culture medium in this study. The formula and a convenient method of preparation via stock solutions are given in Tables 3-1 and 3-2.

Table 3-1. Macroelements of Knop's Solution

Salt	Molecular Weight	Stock Solution		Final Medium Concentration (gm/L)
		Concentration (gm/L)	Use (ml/L)	
MgSO ₄ · 7H ₂ O	246.5	50.0	50.0	2.50
KNO ₃	101.0	25.0	50.0	1.25
KH ₂ PO ₄	136.1	25.0	20.0	0.50

Table 3-2. Hutner-EDTA Microelements for Knop's Solution

Salt	Molecular Weight	Stock Solution		Final Medium Concentration (mg/L of Element)
		Concentration (gm/L)	Use (ml/L)	
CaCl ₂	111.0	8.35	10.0	30
H ₃ BO ₃	61.8	11.42	10.0	20
FeSO ₄ · 7H ₂ O	278.0	4.98	10.0	10
ZnSO ₄ · 7H ₂ O	288.0	8.82	10.0	20
MnCl ₂ · 14H ₂ O	198.0	1.44	10.0	4
MoO ₃	144.0	0.71	10.0	4
CuSO ₄ · 5H ₂ O	250.0	1.57	10.0	4
Co(NO ₃) ₂ · 6H ₂ O	291.0	0.49	10.0	1

This culture medium was prepared by dissolving 0.50 gram of purified ethylene diamino tetra acetic acid (EDTA) in 800 ml of boiling

water, cooling, adding the stock solutions of the macroelements and the Hutner-EDTA microelements, adjusting the final pH to 7.0 by KOH.

In this culture medium, trace elements are stabilized by the use of the chelating agent, EDTA, which forms easily reversible complexes and therefore effectively buffer most of the trace elements. Ethylene diamine tetra acetic acid is relatively inert to other microorganisms and thus can even be used in unsterilized media. This culture medium can support a growth of Chlorella to 2 gm/L dry weight without limitation (56).

Algal Source

An Emerson strain of bacteria-free, experimentally reproducible cultures of Chlorella pyrenoidosa was obtained from Dr. Jack E. Myers, Professor of Zoology and Botany and Director, Laboratory of Algal Physiology, The University of Texas. These algal cultures had a density of 1.0 gm/L dry weight, or equivalent to 3.8 cmm/ml packed cell volume, and had a chlorophyll content of 38 mg/L.

The idea of a steady-state device, called a continuous culture apparatus, and the method used for maintaining the experimentally reproducible algal cultures at a given density were described by Myers (57) and is summarized in Appendix III.

Selected Organic Compounds

Thirty-three phenolic compounds and eight pesticides were investigated. The phenolic-type compounds selected in this study included those with mono- and poly- substituted groups such as -Br, -Cl, -CH₃, -NO₂, -NH₂, -OH. The pesticides selected included DDT,

lindane, 2,4-D, sodium salt of 2,4-D, 2,4,5-T, Dow sodium TCA, Dow Esteron 99 and Dow Formula 40.

Most of the compounds selected are relatively non-volatile as they have very low vapor pressures. Also, these selected compounds have at least a certain degree of water solubility. The volatility of phenol was examined in a test. Phenol was dissolved in distilled water at a concentration of 3000 mg/L and then this solution was aerated for a period of three days. At the end of the third day the phenol concentration in the solution was determined by 4-amino-antipyrine method (68). The results showed that there was almost no loss of phenol in the solution.

The structural formula, certain physical constants including molecular weight, melting point, specific gravity, vapor pressure and water solubility of these selected organic compounds are listed in Table 3-3.

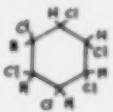
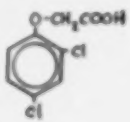
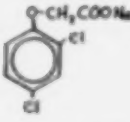
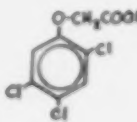
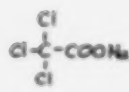
Table 3-3. Structural Formula and Certain Physical Constants of the Selected Organic Compounds

Compounds	Structural Formula	Molecular Weight	Melting Point (°C)	Specific Gravity	Vapor Pressure (°C for 1 mm Hg)	Solubility in Water (gm/100 cc.)
Phenol		94.11	43	1.071	40.1	8.2 at 15°C
o-Bromophenol		173.02	5.6	1.492		slightly
m-Bromophenol		173.02	33			slightly
p-Bromophenol		173.02	66.4	1.840		1.4 at 15°C
o-Chlorophenol		128.56	8.7	1.241	12.1	2.85 at 20°C
m-Chlorophenol		128.56	32.8	1.268	44.2	2.6 at 20°C
p-Chlorophenol		128.56	43	1.265	49.8	2.7 at 20°C
2,4-Dichlorophenol		163.01	45	1.383	53.0	0.45 at 20°C
2,4,5-Trichlorophenol		197.46	66			slightly

Table 3-3. Structural Formula and Certain Physical Constants of the Selected Organic Compounds (Continued)

Compounds	Structural Formula	Molecular Weight	Melting Point (°C)	Specific Gravity	Vapor Pressure (°C for 1 mm Hg)	Solubility in Water (gm/100 cc.)
2,4,6-Trichlorophenol		197.46	68	1.490	76.5	0.09 at 25°C
Pentachlorophenol		266.35	174	1.978		0.003
o-Cresol		108.14	30	1.047	38.2	2.5
m-Cresol		108.14	11.1	1.034	52.0	2.35
p-Cresol		108.14	34.8	1.035	53.0	2.40
2,4-Xylenol		122.16	25	1.036	51.8	slightly
2,5-Xylenol		122.16	74.5		51.8	slightly
2,6-Xylenol		122.16	48			slightly
3,4-Xylenol		122.16	64.5	1.023	66.2	slightly
3,5-Xylenol		122.16	68		62.0	slightly
o-Nitrophenol		139.11	44.9	1.485	49.3	0.21 at 20°C
m-Nitrophenol		139.11	97	1.280		1.35 at 20°C
p-Nitrophenol		139.11	114	1.279		1.6 at 25°C
2,4-Dinitrophenol		184.11	115	1.683		0.5 at cold or 5 at hot slightly
4,6-Dinitro-o-cresol		198.14	85			
o-Aminophenol		109.12	174	1.328		1.7 at 0°C
m-Aminophenol		109.12	123			2.6 at 0°C
p-Aminophenol		109.12	186			1.1 at 0°C
2-Amino-1-phenol-4-sulfonic acid		189.20	270			2 at 14°C
Catechol		110.11	105	1.371		45 at 20°C
Resorcinol		110.11	111	1.272	108.4	123 at 20°C
Hydroquinone		110.11	170	1.328	132.4	8 at 20°C
Pyrogallol		126.11	132.8	1.453		62 at 20°C
Phloroglucinol		126.11	217			1 at 20°C

Table 3-3. Structural Formula and Certain Physical Constants of the Selected Organic Compounds (Continued)

Compounds	Structural Formula	Molecular Weight	Melting Point (°C)	Specific Gravity (C for 1 mm Hg)	Vapor Pressure (mm Hg)	Solubility in Water (gm/100 cc)
DDT (1,1,1-Tri-chloro-2,2-di-(p-chlorophenyl)-ethane)		354.5	104			relatively insoluble
Lindane (Gamma-BHC)		290.9	112			0.001
2,4-D (2,4-Dichloro-phenoxyacetic acid)		221.0	139			0.044
Sodium 2,4-D		243.0	139			soluble
2,4,5-T (2,4,5-Tri-chlorophenoxy-acetic acid)		255.5	153			0.02
Sodium TCA* (Sodium salt of trichloroacetic acid)		185.4				soluble
Esteron 99*	Containing 41% of the propylene glycol butyl ether ester of 2,4-D and 59% of inert ingredients.					soluble
Formula 40*	Containing 65.8% of the alkanolamine (of the ethanolamine or isopropanolamine series) of 2,4-D and 34.2% of inert ingredients.					soluble

* Products of The Dow Chemical Company.

Effect of Initial Algal Mass on the Toxicity

A test was conducted to determine if the different quantities of initial algal mass present in the test tubes would affect the relative toxicity of a compound. In this connection, fifteen test tubes were divided into three equal groups, all of which contained 20 ml of Knop's solution plus a phenol concentration of 200 mg/L in each tube. Then three different quantities of algal source culture suspension, 5 ml, 10 ml, and 15 ml, were centrifuged and added separately into each tube of the three groups. Therefore, these three groups of tubes prepared in this manner contained the same concentration of phenol, but different quantities of initial algal cell mass. The initial concentrations of chlorophyll in these three groups of algal suspensions were 9.7, 19.4 and 29.1 mg/L, respectively.

Another fifteen tubes were also used as the control which were prepared in the same manner except that there was no phenol added. The results of the test are shown in Table 3-4 and Fig. 3-4.

Table 3-4. Effect of Initial Algal Mass on the Toxicity

Group	Initial Algal Mass (mg/L Chlorophyll)	Phenol Concentration (mg/L)	Chlorophyll (mg/L)		
			After 1 Day	After 2 Days	After 3 Days
I	9.7	Control 200	25.6	65.6	64.0
			14.8	25.2	36.0
II	19.4	Control 200	46.8	82.0	61.0
			29.2	46.0	56.0
III	29.1	Control 200	68.0	98.0	67.2
			44.4	62.0	59.0

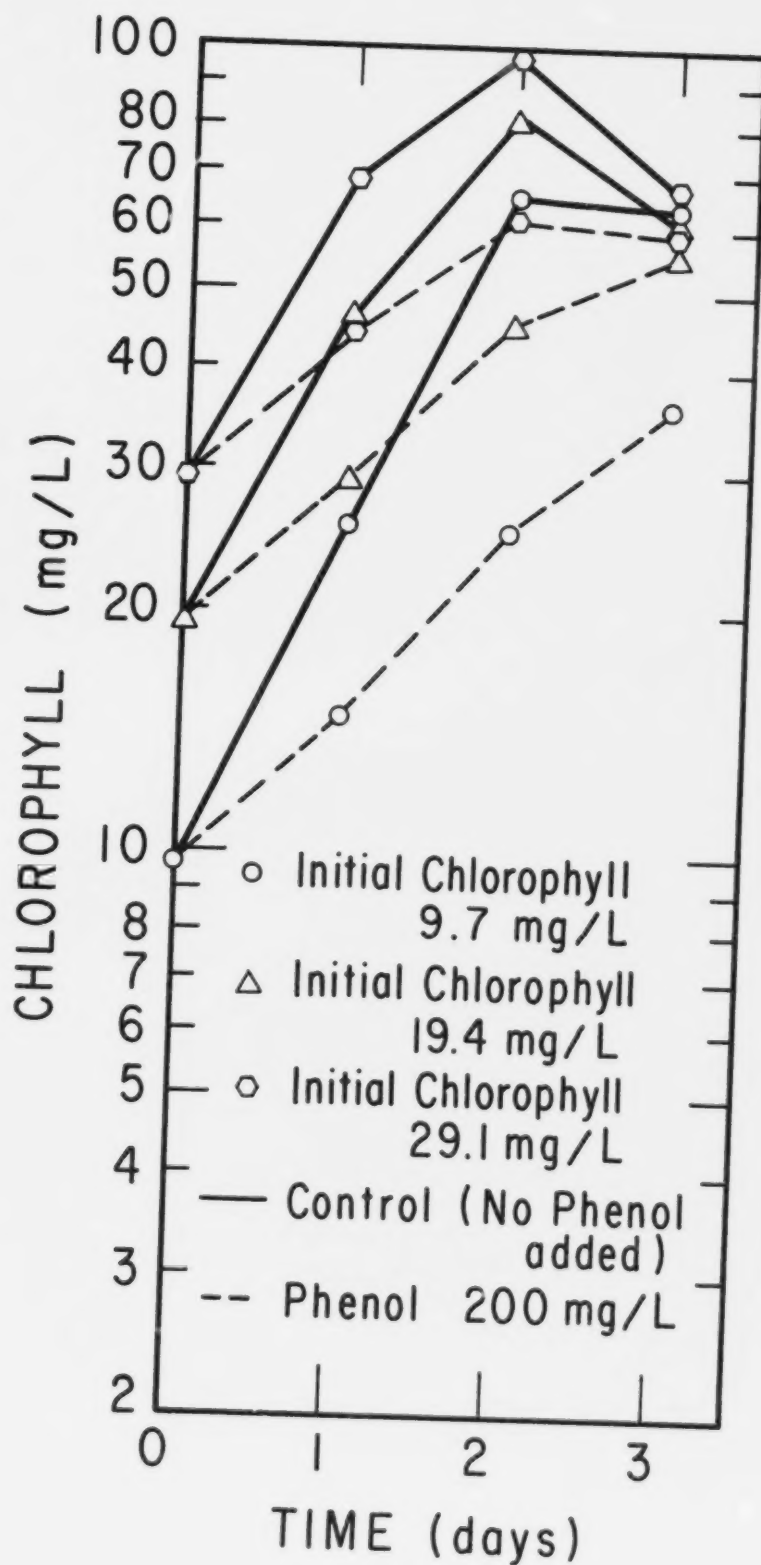


FIG. 3-4. EFFECTS OF INITIAL ALGAL MASS ON THE TOXICITY

From the slopes of the curves, it is seen that the rates of chlorophyll synthesis in the control tubes of all three groups were the same during the first 24 hours. For the next 24 hours, the control tubes of Group I which contained a lowest initial chlorophyll concentration of 9.7 mg/L showed the same exponential rate of chlorophyll synthesis as the first day. Apparently, in this Group I the inorganic nutrients in the culture medium could sufficiently provide an algal growth for a period of two days without any limitation.

On the other hand, for the control tubes of the other two groups which had higher concentrations of initial chlorophyll of 19.4 and 29.1 mg/L, the rates of chlorophyll synthesis could no longer maintain the same exponential rate as the first day. Notably, this was due to the limitation of the general depletion of the inorganic nutrients in the culture medium by the dense algal growth during the first day. For the third day period, all control tubes of the three groups showed decreases in chlorophyll content.

As a contrary to the controls, the rates of chlorophyll synthesis among the three groups of test tubes, which contained 200 mg/L of phenol but with different amounts of initial algal mass, were essentially the same and maintained at a lower exponential rate than that of the control during the first two days. The lower exponential rate of chlorophyll synthesis was due to the toxic effect of phenol.

During the third day, the chlorophyll concentrations in the first two groups containing lower initial chlorophyll contents of 9.7 and 19.4 mg/L still showed similar exponential increase.

However, for the third group which had a highest initial algal mass, the chlorophyll content decreased during the third day. Probably, this was also due to the depletion of inorganic nutrients in the culture medium.

From this test, it is obvious that the different amounts of initial algal mass present in the test tubes of the three groups do not substantially affect the rate of chlorophyll synthesis provided there is no limitation of the inorganic nutrients in the culture medium. Similarly, it is also concluded that the relative toxicity of a compound is not affected by the quantity of initial algal mass.

Difference in Toxicities between Conditions of Continuous Illumination and Alternative Light and Dark

An experiment was also conducted to evaluate the difference in toxicities of phenol under varied environmental conditions, namely, continuous illumination and alternative light and dark.

Two separate tests were made in this investigation. The first test was to evaluate the toxicity of phenol under an environment of continuous illumination for a period of three days. The preparation of test tubes and the test procedure were exactly the same as the routine toxicity test. Seven different concentrations of phenol, ranging from 50 mg/L to 1500 mg/L, were used. Changes of chlorophyll content in algal suspensions were determined every 24 hours. The test data are shown in Table 3-5 and Fig. 3-5.

The second test was to evaluate the toxicity of phenol under an environment of alternative light and dark condition. The test procedure was essentially the same as the first test except that the

illumination was set under a condition of alternative 12 hours of light and 12 hours of dark. The light was switched on at 8:00 AM and switched off at 8:00 PM. Chlorophyll changes in algal suspensions were determined every 12 hours at which the illumination was changed. The test period was 4 days which gave a total illumination period of 48 hours. At the end of the fourth day the chlorophyll concentration in the control started to decrease, probably due to the depletion of inorganic nutrients. Therefore, any test period longer than 4 days was considered impractical. The results are shown in Table 3-6 and Fig. 3-6.

Table 3-5. Chlorophyll Synthesis in the Presence of Phenol under Continuous Illumination

Concentration of Phenol (mg/L)	Chlorophyll (mg/L)			
	After 0 Day	After 1 Day	After 2 Days	After 3 Days
Control	9.8	27.2	74.4	68.0
50	9.8	21.4	44.0	54.0
100	9.8	18.4	37.2	47.2
500	9.8	9.6	11.0	12.0
750	9.8	7.6	7.3	5.6
1,000	9.8	7.3	5.6	3.2
1,250	9.8	5.8	2.8	1.6
1,500	9.8	4.4	0	0

Table 3-6. Chlorophyll Synthesis in the Presence of Phenol under Alternative Light and Dark

Concentration of Phenol (mg/L)	Chlorophyll (mg/L)							
	After 0 Day	After 1 Day		After 2 Days		After 3 Days		After 4 Days
		Light	Dark	Light	Dark	Light	Dark	
Control	9.8	17.8	20.0	34.0	40.0	66.0	68.0	66.0 60.0
50	9.8	13.8	16.0	22.0	25.4	34.0	40.0	52.0 49.8
100	9.8	11.2	15.0	19.5	21.0	27.0	29.2	39.5 39.0
500	9.8	9.8	11.0	12.0	13.6	14.0	15.6	14.5 15.5
750	9.8	8.8	9.0	8.0	9.6	7.6	9.2	7.6 8.8
1,000	9.8	8.4	8.8	6.8	7.4	6.4	7.2	6.6 7.4
1,250	9.8	7.5	8.0	4.4	6.0	3.6	5.2	3.2 4.0
1,500	9.8	6.4	7.2	2.0	2.8	0	0	0 0

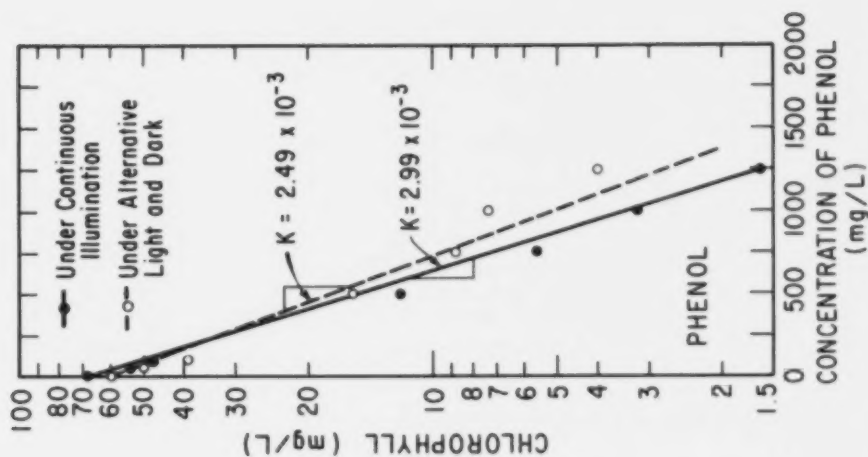


FIG. 3-7. DIFFERENCE IN TOXICITIES OF PHENOL BETWEEN CONDITIONS OF CONTINUOUS ILLUMINATION AND ALTERNATIVE LIGHT AND DARK

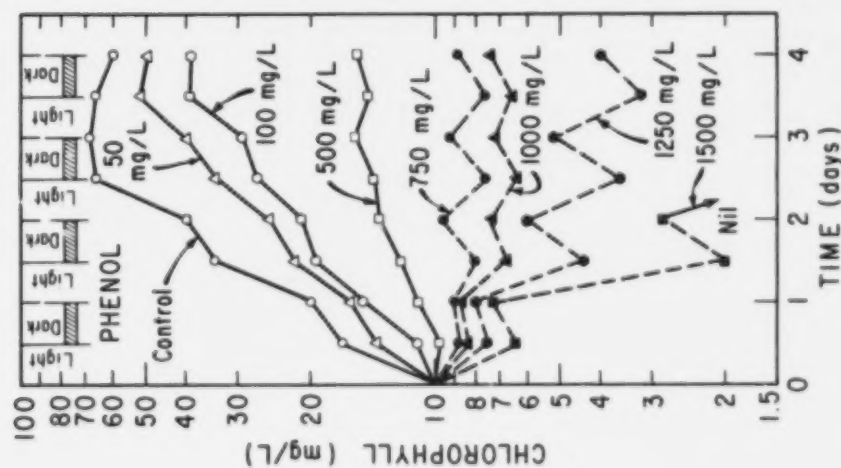


FIG. 3-6. CHLOROPHYLL SYNTHESIS IN THE PRESENCE OF PHENOL UNDER ALTERNATIVE LIGHT AND DARK

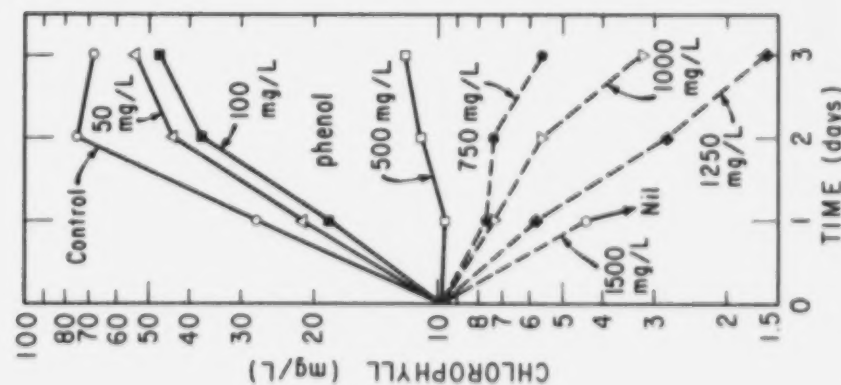


FIG. 3-5. CHLOROPHYLL SYNTHESIS IN THE PRESENCE OF PHENOL UNDER CONTINUOUS ILLUMINATION

From Fig. 3-6 it is seen that the rates of chlorophyll synthesis in the tubes which had a phenol concentration of no more than 100 mg/L were higher during the light periods than during the dark. However, if phenol concentrations were more than 500 mg/L the chlorophyll concentrations showed a decrease after each period of light illumination and then a slight increase after each period of dark. Probably, this was due to the combined effects of decreasing the chlorophyll synthesis and increasing chlorophyll destruction in algal cells when they were illuminated under such unfavorable conditions. Light quantum might stimulate algal cells to be more susceptible to the toxic effects of phenol at a high concentration and hence the chlorophyll synthesis was impaired. Also, chlorophyll might be more easily bleached when algal growth and photosynthetic processes were impeded by the exposure of cells to unfavorable conditions.

If Figs. 3-5 and 3-6 are replotted in such a manner that the concentrations of phenol are plotted as abscissa and the corresponding chlorophyll concentrations are plotted as ordinate in a logarithmic scale, a straight-line relationship, which is similar to the first-order reaction, exists between the increase of phenol and the exponential decrease of chlorophyll in algal suspension. This straight-line relationship is shown in Fig. 3-7.

From Fig. 3-7 it becomes obvious that the slope of the straight line for a certain compound reflects the relative toxicity of that compound to algae. The calculation of the slope K , which is defined as the toxicity constant for a certain compound, is

illustrated below:

Let Q_1 represent the chlorophyll concentration which results from the addition of a compound having a concentration C_1 ; and let Q_2 represent the chlorophyll concentration resulting from the addition of the same compound having a concentration C_2 . Then from the straight line of a first-order relationship, it is possible to write Eq. 3-1.

$$\frac{Q_2}{Q_1} = e^{-K \cdot (C_2 - C_1)} \quad \dots\dots\dots \text{Eq. 3-1}$$

For instance, as shown in Fig. 3-7, in the solid line representing the chlorophyll synthesis in the presence of different concentrations of phenol under a condition of continuous illumination and when there was no phenol added ($C_1 = 0 \text{ mg/L}$), the chlorophyll concentration (Q_1) was 68 mg/L. Also, when there was a concentration $C_2 = 1000 \text{ mg/L}$ of phenol added, the chlorophyll concentration was $Q_2 = 3.4 \text{ mg/L}$. By substituting these numbers into Eq. 3-1 it is possible to calculate K .

$$\frac{Q_2}{Q_1} = \frac{3.4}{68.0} = e^{-K \cdot (1000 - 0)}$$

$$\therefore K = 2.99 \times 10^{-3}$$

Similarly, the K value for the dash line which represents the chlorophyll synthesis in the presence of different concentrations of phenol under a condition of alternative light and dark is 2.49×10^{-3} . Therefore, it appears that cells of Chlorella pyrenoidosa may be slightly more susceptible to the toxic effect of phenol under an environment of continuous illumination than under an alternative light and dark condition.

Comparison between Chlorophyll Synthesis and Algal Growth

There are several methods commonly used to estimate an algal growth such as gravimetric measurement of the algal dry weight, volumetric measurement of the packed cells, turbidimetric measurement of the density of algal cell suspensions or statistical microscopic counting of the number of algal cells. The selection of a proper method for evaluating an algal growth depends on the specific purpose of a problem.

The main purpose of this research was to investigate the suppression of the photosynthetic process by the organic compounds. Since the photosynthetic activity of Chlorella is generally proportional to the chlorophyll content in the cells (23), the changes of chlorophyll content in the test suspension were used as an estimation of the relative magnitude of toxicity. Also, the chlorophyll extraction eliminated the possible interfering effects of foreign substances during the photometric determination of the chlorophyll concentration.

A test was made to compare the chlorophyll synthesis and the algal growth in control tubes during the course of a 5-day test period. The test procedure was exactly the same as the routine toxicity test. The chlorophyll concentration and the algal dry weight of the control suspensions were measured every day. The results are shown in Table 3-7 and Fig. 3-8. The percentage of the chlorophyll content on the algal dry weight basis is also shown in the figure.

Noticeably, both the chlorophyll concentration and algal dry weight showed the exponential increase in the first two days and reached a value, respectively, of 74.6 and 2740 mg/L. Thereafter,

the chlorophyll concentration started to decrease and dropped to 48.2 mg/L at the end of the fifth day, while the algal dry weight still kept a slight increase and reached a maximum value of 3550 mg/L at the end of the fifth day. Therefore, it shows that the synthesis of algal cell material does not necessarily always accompany the same rate of chlorophyll synthesis within the cells.

Table 3-7. Comparison between Chlorophyll Synthesis and Algal Growth

Description	After 0 Day	After 1 Day	After 2 Days	After 3 Days	After 4 Days	After 5 Days
Chlorophyll (mg/L)	9.2	24.6	74.6	64.8	56.2	48.2
Algal Dry Wt. (mg/L)	246	880	2740	2940	3450	3550
% of Chloro- phyll on dry wt. basis	3.72	2.80	2.72	2.20	1.63	1.36

The initial percentage of the chlorophyll content in the test algal suspension was 3.72 on the cell dry weight basis and gradually decreased to a value of 1.36 after five days. The decrease of chlorophyll percentage in algal cells was probably due to the depletion of some inorganic nutrients such as nitrate (23) and iron (62) in the culture medium.

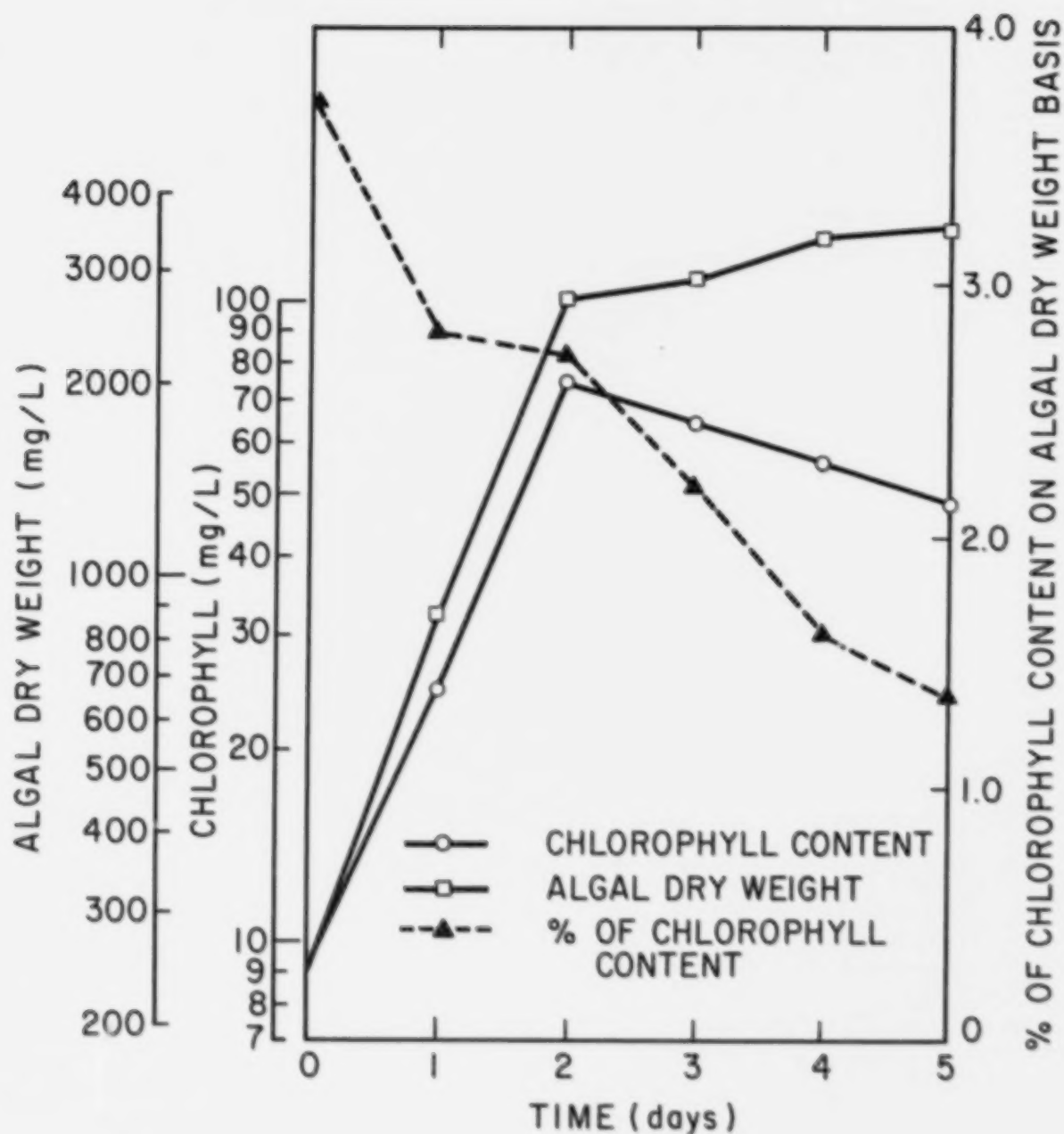


FIG. 3-8. COMPARISON BETWEEN CHLOROPHYLL SYNTHESIS AND ALGAL GROWTH

Evaluation of Results

The term "toxicity" has various meanings to different people when applied in different subjects. In general, "toxicity" refers to the fact that a test organism fails to survive or the metabolic activity of a test organism is reduced due to the presence of certain substances which either interfere with some enzymatic system and thus block the metabolism, or merely cause an unfavorable environmental condition and hence upset an optimum growth. For the purposes of evaluation and interpretation of the toxicity data of selected organic compounds to algae, the meaning of toxicity must be first defined.

In this study the term "toxicity" is defined as any physical, chemical, or biochemical change, whichever may occur within cells of Chlorella pyrenoidosa upon the addition of the tested organic compound in conjunction with a reduction of chlorophyll content. Hence, relative toxicity of a compound reflects a relative magnitude of the suppression of chlorophyll synthesis.

The results of the toxicity of phenolic-type compounds are presented in groups according to their substituted groups and molecular structures. The toxicity data of eight pesticides are last presented.

The entire test data are shown in Tables 3-8 through 3-16. The chlorophyll synthesis as a function of time at different concentrations of each compound is also plotted.

For most of the compounds studied, the toxic effects of a test organic compound generally exhibit a first-order reaction in

reducing the chlorophyll in algal cells. This phenomenon is demonstrated by plotting the chlorophyll concentrations at the end of the test as ordinate in a logarithmic scale and the corresponding organic concentrations as abscissa, there exists a straight-line relationship for most of the selected compounds.

Phenol

Phenol is recovered from coal tar, and today considerable amounts are manufactured synthetically. It is used extensively in the synthesis of organic products, particularly phenolic-type resins. It occurs as a component of industrial wastes from the coal-gas, coal-coking, and petroleum industries as well as in a wide variety of industrial wastes from processes involving the use of phenol as a raw material.

The oldest known disinfectant is phenol, which was first introduced as a bactericidal agent in surgery. The bactericidal action of phenol is believed to be due to the coagulation of bacterial protein (17). It is also relatively toxic to people, the fatal dose for an adult being 8 to 15 grams.

Since phenol is the first chemical used as a bactericide, efficiencies of other chemicals have been rated in terms of "phenol coefficients," i.e., relative disinfecting power with respect to phenol (17).

The relative toxicity of phenol is previously already shown in Table 3-5 and Figs. 3-5 and 3-7. As shown in Fig. 3-5, the chlorophyll concentration was 68 mg/L in the control and was

reduced to 48 mg/L in the presence of 100 mg/L of phenol. This was equivalent to approximately a 30 percent reduction of chlorophyll synthesis in the presence of 100 mg/L phenol. If phenol concentration was raised to 500 mg/L, there was no substantial chlorophyll synthesis in algal suspensions during the course of the three-day test period. When phenol concentrations were more than 750 mg/L, chlorophyll destruction in algal suspensions was greater than chlorophyll synthesis, if any of which still remained. At a concentration of 1500 mg/L of phenol, chlorophyll in algal cells disappeared completely at the end of the second day. The above toxicity data are generally in good agreement with those found by Thirumurthi (73). However, Korsep (39) found that relative magnitudes of toxicity of phenol at concentrations of 100 mg/L and 500 mg/L were not much different to Chlorella pyrenoidosa.

Huang (34) found that in an algae-bacteria combined system the rate of chlorophyll synthesis in the presence of 200 mg/L of phenol was slightly less than 50 percent of that of control. Apparently, the toxicity of phenol to algae was not materially affected by the presence of bacteria if phenol was not substantially degraded by the bacterial system.

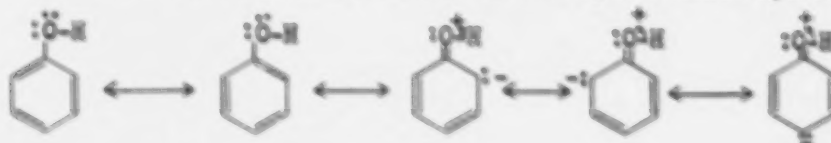
Ukeles (74) found that a Chlorella sp. in the presence of 100 mg/L and 300 mg/L of phenol had, respectively, only a growth of 84 percent and 63 percent of that of control. If the concentration of phenol was raised to 500 mg/L, there was completely no algal growth. The discrepancy between Ukeles' data and the results obtained in this study was probably attributed to the different species of algae, culture media and culturing methods used in these studies.

Using the plating technique, the toxicity of phenol to a blue-green alga, Anacystis nidulans was also investigated by Moore (55). The results indicated that a 1.5 percent of phenol solution killed A. nidulans very rapidly; however, at concentrations of 0.5 percent and below, little killing was observed up to a period of 30 minutes. The resistance of A. nidulans to the treatment of phenol was found greater than that of E. coli.

From Fig. 3-7, it is seen that the decrease in chlorophyll concentration is generally logarithmic to the concentration of phenol. The toxicity constant, K , was found to be 2.99×10^{-3} . This indicated that the chlorophyll syntheses were only approximately $2/3$, $1/2$, and $1/3$, respectively, of the control when the phenol concentrations were 100 mg/L, 200 mg/L, and 400 mg/L.

Mono-Halogenated Phenols

Phenols normally exist as the following resonance hybrids:



Therefore, the presence of the hydroxyl group in phenol activates the formation of o- and p-halogenated phenols. For example, chlorophenols are readily produced when phenol contaminated water is chlorinated in the purification process. These halogenated phenols are very distasteful even in the range of few parts per billion.

The above resonance hybrids also explain the weak acidic characteristic of phenol since the oxygen atom acquires a positive

charge and so attracts the electron pair of the O:H bond, thereby facilitating the release of a proton.

Six mono-halogenated phenols were selected for the toxicity study. They were o-, m-, and p-substituted bromophenols and chlorophenols. The toxicity data and the toxicity constants of these six compounds are included in Tables 3-8 and 3-9, also in Figs. 3-9 through 3-13.

The results indicated that at a concentration of 10 mg/L or below, there was completely no toxic effect on the chlorophyll synthesis for all of these six halogeno-phenols.

Among the three bromophenols, the ortho derivative was the least toxic to Chlorella pyrenoidosa, the para derivative the next, while the meta-bromophenol was the most toxic. As shown in Fig. 3-9, in the presence of 50 mg/L of o-bromophenol the chlorophyll concentration still showed a continuous, steady increase, even though at a lower rate than the control, and reached a value of 48 mg/L, which was equivalent to 78 percent of the control; however, at a higher concentration of 100 mg/L the chlorophyll synthesis began to lag for the first day and then increased steadily to 29.2 mg/L at the conclusion of the test; if the concentration was raised to 250 mg/L or higher, the rate of chlorophyll destruction became greater than that of chlorophyll synthesis.

Meta- and para-bromophenols had greater toxicities than ortho-bromophenol, the m-derivative being a bit greater than the p-derivative. As shown in Fig. 3-10, the chlorophyll contents still showed a steady increase for both compounds at a concentration of 50 mg/L.

However, the chlorophyll concentrations were not substantially changed during the course of the three-day test period if the concentration was raised to 100 mg/L for either m- or p-bromophenol, and completely diminished within two days at a concentration of 250 mg/L.

Table 3-8. Test Data for Bromophenols

Compound	Concentration of Compound (mg/L)	Chlorophyll (mg/L)			
		After 0 Day	After 1 Day	After 2 Days	After 3 Days
o-Bromophenol	Control	9.6	24.4	76.8	62.0
	10	9.6	24.0	74.4	64.0
	50	9.6	20.0	30.0	48.0
	100	9.6	10.0	14.8	29.2
	250	9.6	9.2	7.6	6.8
	500	9.6	0	0	0
m-Bromophenol	Control	9.6	24.4	76.8	62.0
	10	9.6	25.2	76.8	63.2
	50	9.6	18.4	24.4	32.0
	100	9.6	9.2	9.6	7.6
	250	9.6	3.6	0	0
	500	9.6	0	0	0
p-Bromophenol	Control	9.6	24.4	76.8	62.0
	10	9.6	24.4	76.0	62.0
	50	9.6	19.2	27.6	37.6
	100	9.6	10.0	9.6	9.2
	250	9.6	5.6	1.6	0
	500	9.6	0	0	0

The relative magnitudes of toxicity of the three chlorophenols as a function of their substituted position showed a striking similarity with those of bromophenols. However, chlorophenols generally had slightly smaller toxicities as compared to the corresponding bromophenols. For example, at a concentration of 100 mg/L of p-chlorophenol the chlorophyll concentration still increased slowly and reached 13.6 mg/L

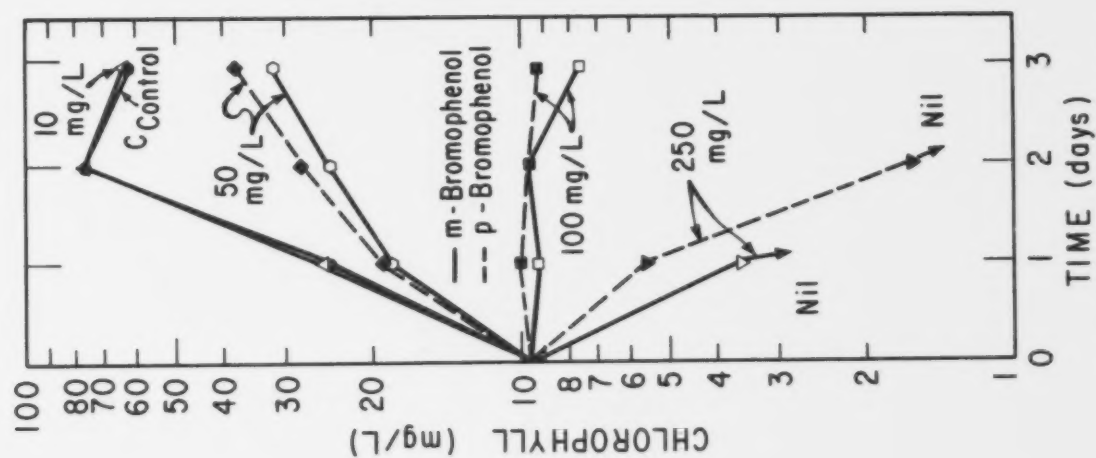


FIG. 3-9. EFFECTS OF
o-BROMOPHENOL

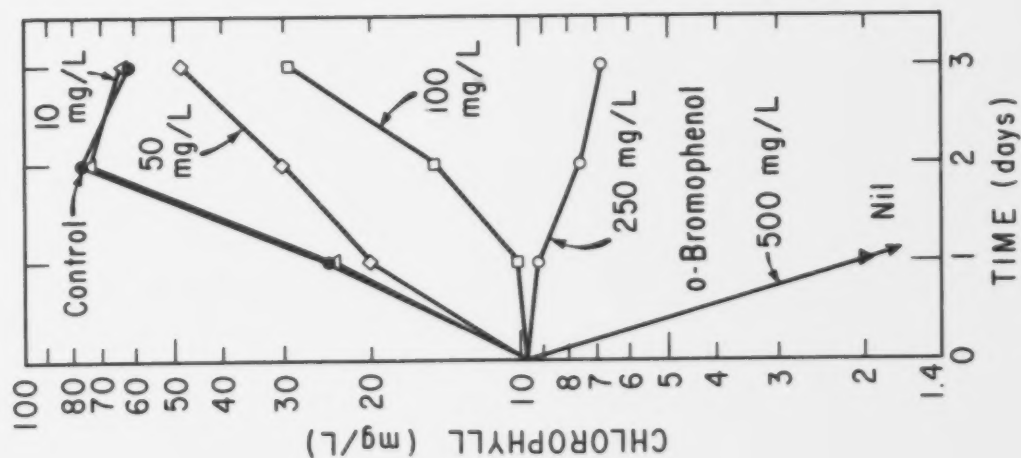


FIG. 3-10. EFFECTS OF m-
AND p-BROMOPHENOLS

at the end of the test; also, at a concentration of 250 mg/L of either p- or m-chlorophenol the chlorophyll was not completely eliminated, but having a very low concentration of less than 2 mg/L at the end of the third day. The results are shown in Table 3-9 and Figs. 3-11 and 3-12.

Table 3-9. Test Data for Chlorophenols

Compound	Concentration of Compound (mg/L)	Chlorophyll (mg/L)			
		After 0 Day	After 1 Day	After 2 Days	After 3 Days
o-Chlorophenol	Control	9.6	24.8	74.6	66.8
	10	9.6	25.6	76.8	64.8
	50	9.6	16.8	40.0	52.0
	100	9.6	10.4	28.0	44.0
	250	9.6	9.6	8.4	8.0
	500	9.6	6.4	2.4	1.2
m-Chlorophenol	Control	9.6	24.8	74.6	66.8
	10	9.6	24.8	76.0	64.8
	50	9.6	18.4	22.8	32.0
	100	9.6	10.0	9.4	8.4
	250	9.6	6.0	3.2	1.2
	500	9.6	0	0	0
p-Chlorophenol	Control	9.6	24.8	74.6	66.8
	10	9.6	24.6	75.6	66.8
	50	9.6	20.0	25.2	36.0
	100	9.6	11.6	12.8	13.6
	250	9.6	6.8	4.4	2.0
	500	9.6	2.0	0	0

For these six mono-halogenated phenols tested, the decrease in chlorophyll content was generally logarithmic to the increase of the compound, as shown in Fig. 3-13. The relative toxicity expressed in terms of the toxicity constant, K, of each compound is also shown in the figure.

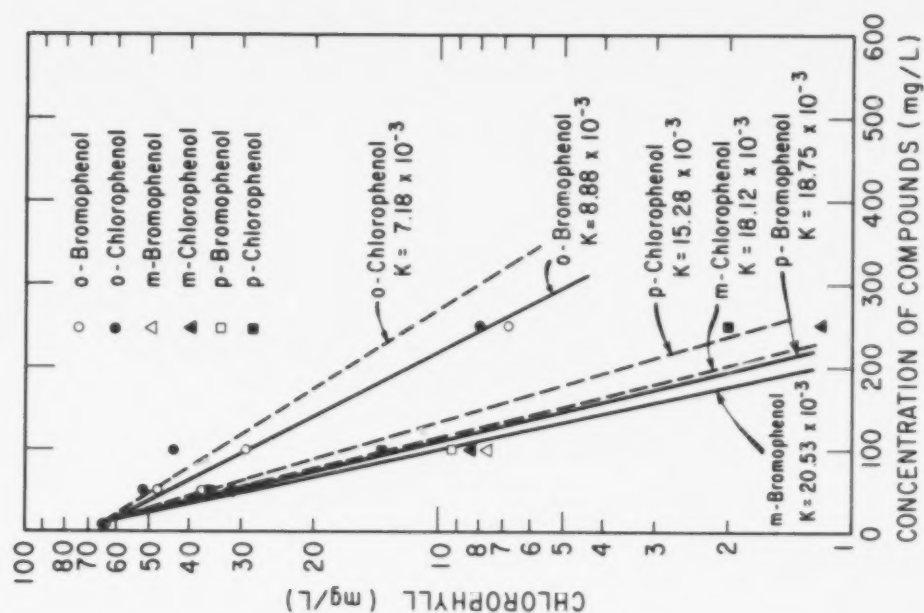


FIG. 3-13. TOXICITY CONSTANTS OF THE SIX MONO-HALOGENATED PHENOLS

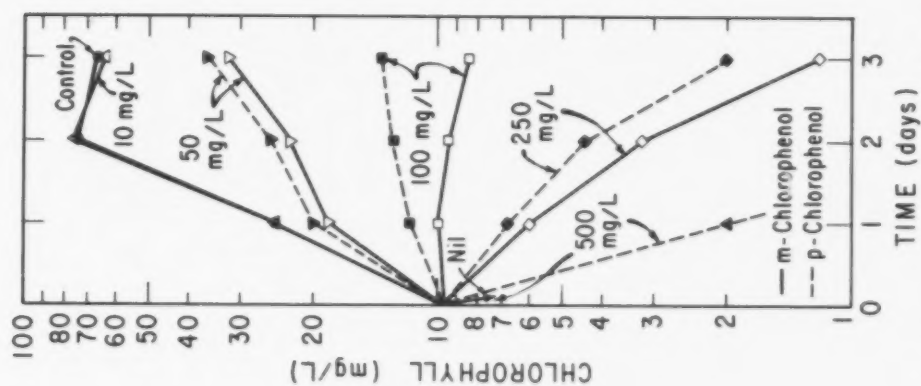


FIG. 3-12. EFFECTS OF m- AND p-CHLOROPHENOLS

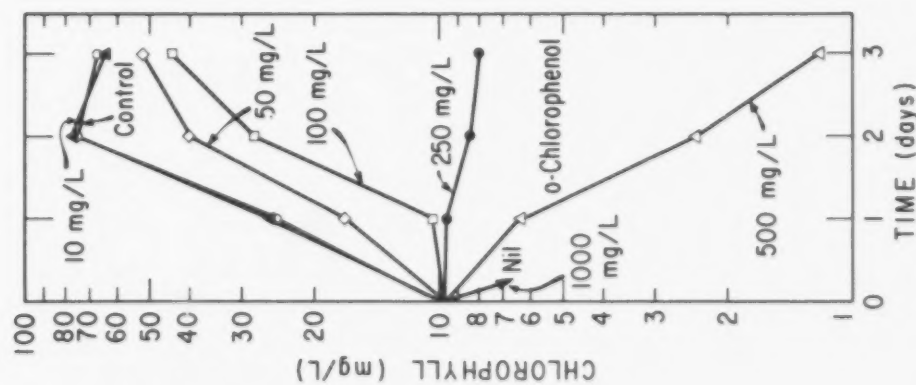
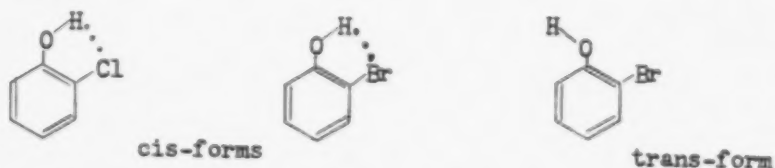


FIG. 3-11. EFFECTS OF o-CHLOROPHENOL

It has been reported by Fieser (17) that introduction of halogen atoms to phenol increases the bactericidal activity of the parent compound itself. The inductive effect of the halogen atom which enhances resonance and thereby increasing their acidity may partly account for the greater toxicity of these halogenated phenols.

The bactericidal activities, expressed as phenol coefficients, of o-, p- and m-chlorophenol to *B. typhosus* were, respectively, 3.6, 3.9, and 7.4 (17). As shown in Fig. 3-13 the phenol coefficients of these three chlorophenols to *Chlorella pyrenoidosa* were found, respectively, to be 2.4 ($= \frac{7.18}{2.99}$), 5.1 ($= \frac{15.28}{2.99}$), and 6.1 ($= \frac{18.12}{2.99}$).

The greater toxicity of bromophenols than that of the corresponding chlorophenols may probably be due to the facts that a.) bromine atom has greater activity than chlorine atom in affecting biological processes; b.) o-bromophenol can exist in the two forms of isomers, cis- and trans-forms, due to the hydrogen bonding while o-chlorophenol can exist entirely in cis-form as shown in the following (18):



Hence, the stronger hydrogen bonding for chlorine may partly suppress the activity of chlorine atom and thus also reduce the toxicity of ortho-chlorophenol. The bromine atom present in the trans-form of ortho-bromophenol is not bonded by hydrogen and thus believed to have higher activity.

In studying the algicidal effect of bromine and chlorine on Chlorella pyrenoidosa, Kott et al. (40) found that bromine and bromamine were primarily algicidal whereas chlorine and chloramine were mainly algistatic. This suggests that bromine has a higher toxic effect than chlorine to Chlorella pyrenoidosa and it is in good agreement with the experimental data obtained in this study as described above. Kott et al. (40) also assumed that the mechanisms of action of these two halogens on Chlorella are not the same.

Tabak, Chambers and Kabler (71) studied the degradation of m- and p-chlorophenols by phenol-adapted bacteria and found that the degradability of the former was less than that of the latter. It seems to be reasonable to assume that for similar halogenophenols with only different substituted position, the relative magnitudes of toxicity to microorganisms are generally consistent with their degrees of resistance to biological degradation.

Poly-Halogenated Phenols

Four poly-chlorinated phenols, i.e., 2,4-dichlorophenol, 2,4,5- and 2,4,6-trichlorophenols and pentachlorophenol, were investigated. The test data are shown in Table 3-10 and Figs. 3-14 through 3-19.

The results indicated that these poly-chlorinated phenols were much more toxic to Chlorella pyrenoidosa than the mono-chlorinated phenols. The lethal dosage was 100 mg/L for 2,4-dichlorophenol and only 10 mg/L for both 2,4,5- and 2,4,6-trichlorophenols within

a period of two days. Pentachlorophenol is relatively insoluble in water, the concentration of this compound shown in Table 3-10 and Fig. 3-16 are expressed as percentage of the saturated solution. It is reported in Lange Chemistry Handbook (41) that the solubility of pentachlorophenol in water is 0.003 gram per 100 c.c. If this solubility value is accurate, then the lethal dosage for pentachlorophenol to Chlorella pyrenoidosa is calculated to be 7.5 $\mu\text{g/L}$, which is equivalent to a concentration of (1/40)% saturated pentachlorophenol solution.

As shown in Fig. 3-14, 2,4-dichlorophenol at a concentration of 50 mg/L or below inhibited the chlorophyll synthesis for the first day, thereafter the chlorophyll synthesis resumed at a moderate rate. If the concentration was raised to 100 mg/L, algal cells became impaired and ceased to function.

The relative magnitudes of toxicity of 2,4,5- and 2,4,6-trichlorophenols were about ten-fold as much as that of 2,4-dichlorophenol. The compound of 2,4,5-trichlorophenol had a slightly higher toxicity as compared with 2,4,6-trichlorophenol. As shown in Fig. 3-15 the chlorophyll synthesis was gradually reduced at a concentration of 5 mg/L for both trichlorophenols, and the chlorophyll was completely eliminated at a concentration of 10 mg/L for both compounds.

Pentachlorophenol was the most toxic compound among the ten halogeno-phenols tested. Even at a concentration as low as 1.5 $\mu\text{g/L}$, which is equivalent to a (1/200)% saturated solution, pentachlorophenol inhibited chlorophyll synthesis for a period of two days. It completely killed algae at a very low concentration of 7.5 $\mu\text{g/L}$.

Table 3-10. Test Data for Poly-Halogenated Phenols

Compound	Concentration of Compound (mg/L)	Chlorophyll (mg/L)			
		After 0 Day	After 1 Day	After 2 Days	After 3 Days
2,4-Dichlorophenol	Control	10.4	24.8	77.0	64.8
	1	10.4	25.6	46.8	60.0
	10	10.4	12.8	36.6	50.2
	25	10.4	9.6	15.6	24.0
	50	10.4	8.0	11.6	12.8
	100	10.4	5.0	1.6	0
2,4,5-Trichlorophenol	Control	10.4	24.8	77.0	64.8
	1.0	10.4	20.0	52.8	56.8
	2.5	10.4	16.0	18.4	26.4
	5.0	10.4	12.2	9.6	8.0
	7.5	10.4	8.8	6.2	2.4
	10.0	10.4	6.2	1.6	0
2,4,6-Trichlorophenol	Control	10.4	24.8	77.0	64.8
	1.0	10.4	24.8	74.4	58.4
	2.5	10.4	18.4	26.0	32.0
	5.0	10.4	15.6	12.8	9.6
	7.5	10.4	11.6	7.2	4.4
	10.0	10.4	8.8	4.4	0
Pentachlorophenol	Control	9.7	26.4	77.6	68.8
	(1/400)%	9.7	11.6	23.6	44.4
	(1/200)%	9.7	7.0	7.0	20.0
	(1/80)%	9.7	4.6	5.2	6.8
	(1/40)%	9.7	2.4	0	0

The characteristics of the first-order reaction exhibited by these four poly-halogenated phenols in inhibiting the chlorophyll synthesis are shown in Figs. 3-17 to 3-19. The chlorophyll synthesis was not materially interfered by both trichlorophenols at a concentration of 1 mg/L. Therefore, a threshold toxic concentration of 1 mg/L was established for both compounds in calculating the toxicity constants. Also, the toxicity constant for pentachlorophenol was calculated by

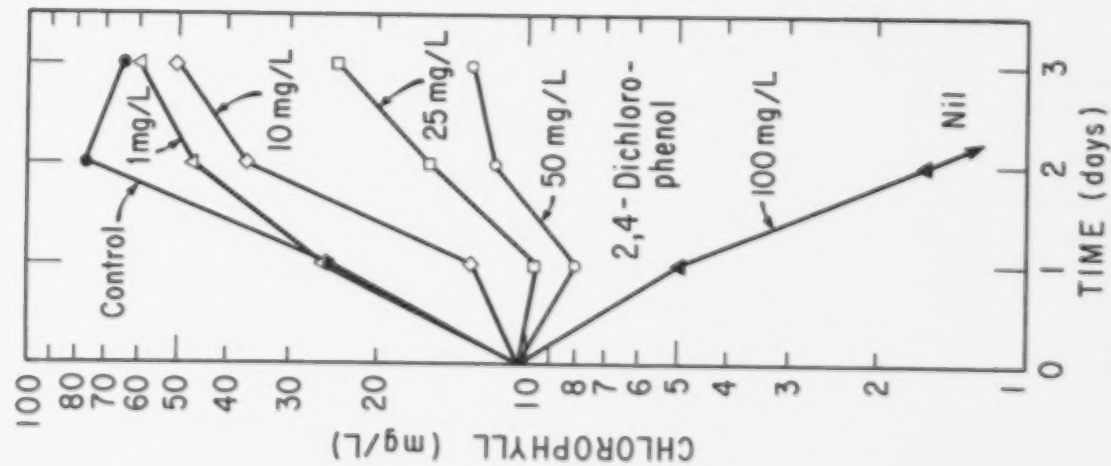


FIG. 3-14. EFFECTS OF 2,4-DICHLOROPHENOL

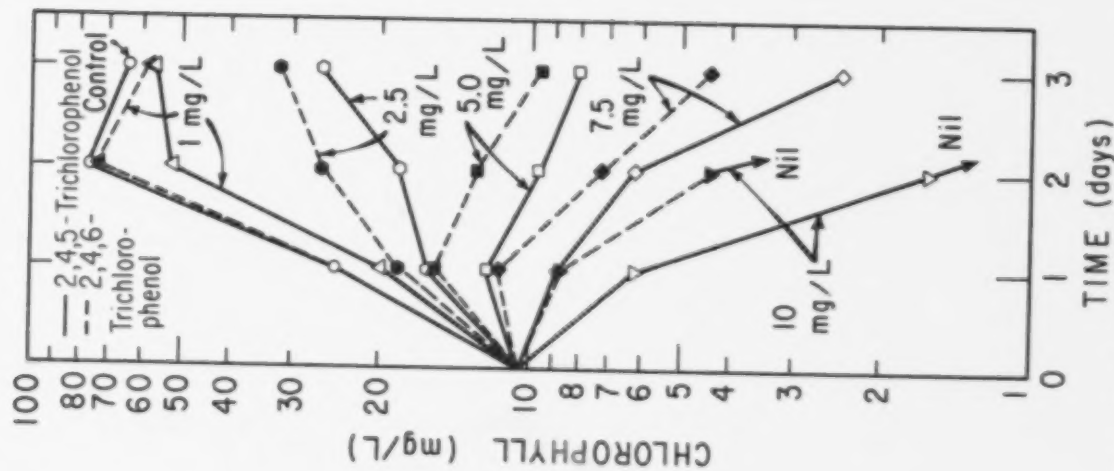


FIG. 3-15. EFFECTS OF 2,4,5- AND 2,4,6-TRICHLOROPHENOLS

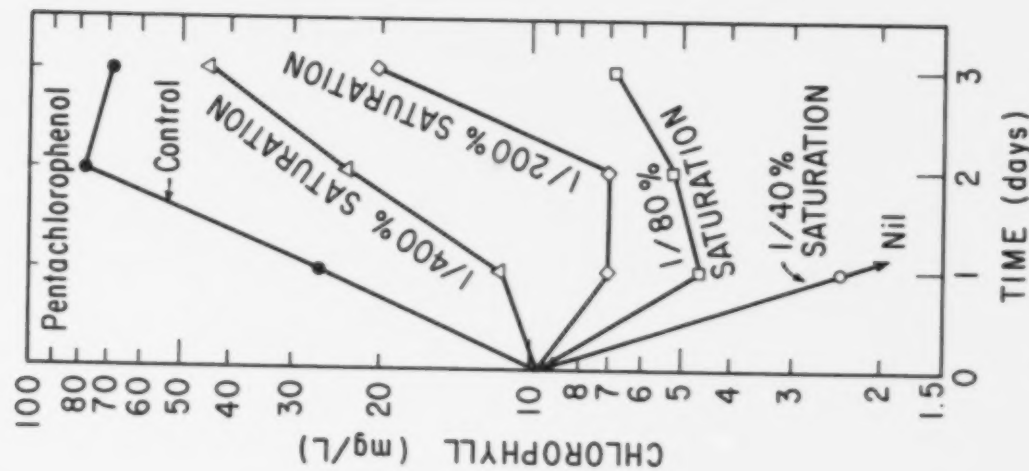


FIG. 3-16. EFFECTS OF PENTACHLOROPHENOL

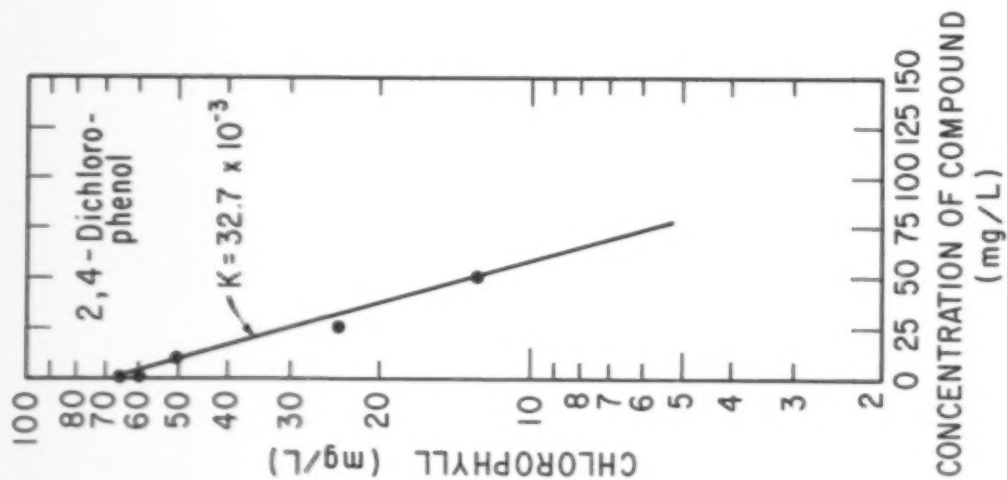


FIG. 3-17. TOXICITY CONSTANT OF 2,4-DICHLOROPHENOL

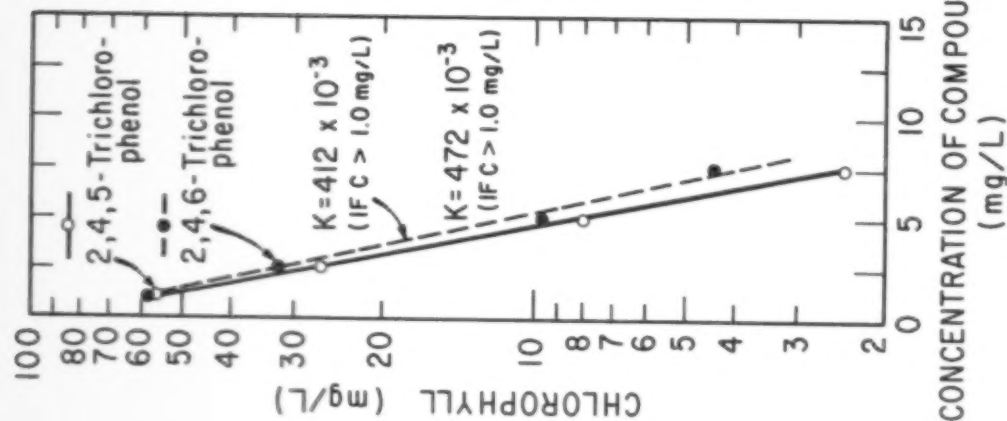


FIG. 3-18. TOXICITY CONSTANTS 2,4,5- AND 2,4,6-TRICHLOROPHENOLS

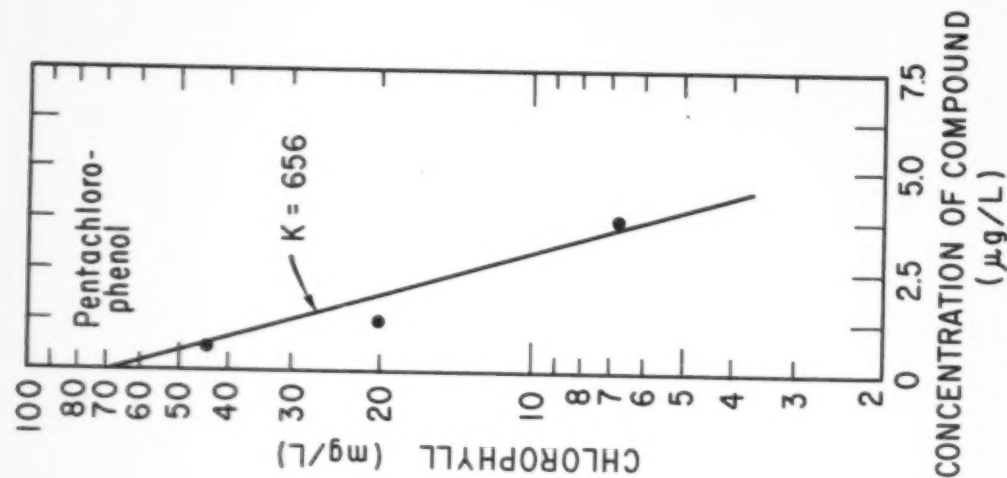


FIG. 3-19. TOXICITY CONSTANT OF PENTACHLOROPHENOL

assuming a saturated solubility of 0.003 gram per 100 c.c. of water for this compound and was found to be a value of 656, which was 220,000 times as high as the toxicity of phenol.

From the toxicity data of the ten halogeno-phenols investigated, it is concluded that the addition of halogen atoms to the parent phenol increases the toxicity to Chlorella pyrenoidosa. The more the halogen atoms added to a compound, the higher the toxicity is. The same relationships have also been reported by Fieser (17) as regard to the bactericidal properties of the halogeno-phenols. However, the drastic decrease in water solubility of these poly-halogenated compounds may sometimes limit their practical values.

Alkyl-Substituted Phenols

Three monomethyl-substituted phenols and five dimethyl-substituted phenols were included in the toxicity study. The monomethyl phenols included o-, m-, and p-cresols while dimethyl phenols included 2,4-, 2,5-, 2,6-, 3,4-, and 3,5-xylenols.

All of these cresols and xylenols are found in coal tar. Cresols are reported to have higher germicidal actions than phenol, but are less toxic to people (17). Therefore, a solution of the cresols stabilized by soap is marketed as Lysol to disinfect the household and sickroom. Cresols are also the major constituents of "creosote" which is used extensively for the preservation of wood. Meta- and p-cresols are present in the wastes of resin and plastics industries.

The toxicity of these eight alkyl-substituted phenols to Chlorella pyrenoidosa are shown in Tables 3-11 and 3-12, also in Figs. 3-20 through 3-25.

Generally, there were no significant differences in relative magnitudes of toxicity between the three cresols, with p-isomer being only a bit higher and m-isomer a bit smaller as compared with the toxicity of o-cresol. The variations of changes of chlorophyll concentrations as a function of time were also very similar for these three cresols, as shown in Figs. 3-20 and 3-21. At concentrations less than 100 mg/L all of the three cresols were not very effective in reducing the chlorophyll synthesis. However, at the concentration of 250 mg/L these three cresols inhibited the chlorophyll synthesis, as the chlorophyll contents showed no substantial changes during the course of three-day test period. If the concentrations of cresols were above 250 mg/L, the destruction of chlorophyll in algal suspensions became more apparent, and at a concentration of 1000 mg/L the chlorophyll completely disappeared within two days of incubation.

Xylenols generally exhibited higher toxicities than cresols. However, at a concentration of 50 mg/L or below, these five xylenols did not exhibit significant toxicity. As shown in Figs. 3-22 to 3-24, the degree of toxicity and the patterns of chlorophyll variations at various concentrations of xylenols were very similar. The compounds of 2,4- and 2,5-xylenols had only slightly higher toxicities than the 3,4- and 3,5-isomers; however, 2,6-xyleneol was the least toxic compound in the group.

At the concentration of 100 mg/L all xylenols except the 2,6-isomer lagged for one day the chlorophyll synthesis, but which

was recovered quickly during the next day. If the concentrations were greater than 250 mg/L, the chlorophyll synthesis became interfered by all of the five xlenols.

Table 3-11. Test Data for Cresols

Compound	Concentration of Compound (mg/L)	Chlorophyll (mg/L)			
		After 0 Day	After 1 Day	After 2 Days	After 3 Days
o-Cresol	Control	9.6	24.4	77.0	62.0
	50	9.6	23.4	63.2	49.6
	100	9.6	21.6	51.4	38.6
	250	9.6	10.4	9.6	9.6
	500	9.6	9.2	8.4	6.8
	750	9.6	7.6	4.4	2.8
	1,000	9.6	2.4	0	0
m-Cresol	Control	9.6	24.4	77.0	62.0
	50	9.6	23.6	64.8	51.6
	100	9.6	22.4	52.4	39.2
	250	9.6	10.4	10.0	10.4
	500	9.6	9.6	8.8	7.6
	750	9.6	8.0	4.8	3.2
	1,000	9.6	4.4	0	0
p-Cresol	Control	9.6	24.4	77.0	62.0
	50	9.6	23.2	63.2	49.6
	100	9.6	21.2	50.8	37.6
	250	9.6	9.6	9.0	9.2
	500	9.6	8.8	8.4	6.0
	750	9.6	7.6	3.2	2.0
	1,000	9.6	1.2	0	0

The toxicity constants of these eight alkyl-substituted phenols are shown in Fig. 3-25. Since the compound of the five xlenols did not exhibit significant toxic effects on algae at concentrations less than 50 mg/L, a threshold toxic concentration of 50 mg/L was established for the five xlenols in the calculation of the toxicity constants.

Table 3-12. Test Data for Xylenols

Compound	Concentration of Compound (mg/L)	Chlorophyll (mg/L)			
		After 0 Day	After 1 Day	After 2 Days	After 3 Days
2,4-Xylenol	Control	9.6	24.0	76.0	64.0
	10	9.6	24.0	74.4	64.8
	50	9.6	19.4	63.6	62.0
	100	9.6	12.0	19.6	30.8
	250	9.6	7.2	5.6	3.6
	500	9.6	2.8	0	0
2,5-Xylenol	Control	9.6	24.0	76.0	64.0
	10	9.6	23.2	74.8	63.2
	50	9.6	18.0	58.0	62.0
	100	9.6	10.4	18.4	32.8
	250	9.6	8.0	6.8	5.6
	500	9.6	5.6	2.8	0
2,6-Xylenol	Control	9.6	24.0	76.0	64.0
	10	9.6	24.0	76.8	64.8
	50	9.6	20.0	64.8	62.4
	100	9.6	15.6	28.0	39.2
	250	9.6	9.6	7.4	8.0
	500	9.6	7.4	4.0	2.8
3,4-Xylenol	Control	9.7	25.6	74.4	64.0
	10	9.7	24.8	72.8	64.8
	50	9.7	20.0	64.8	63.2
	100	9.7	10.4	17.6	32.8
	250	9.7	8.0	7.6	6.8
	500	9.7	4.4	2.4	0
3,5-Xylenol	Control	9.7	25.6	74.4	64.0
	10	9.7	24.4	74.8	64.8
	50	9.7	20.8	66.4	63.8
	100	9.7	10.4	17.0	33.6
	250	9.7	8.0	8.4	8.8
	500	9.7	4.8	1.2	0

From the above toxicity data, it is evident that the addition of a methyl group to the phenol nucleus slightly increases the toxicity. The addition of a second methyl group further increases the toxicity to Chlorella pyrenoidosa. The substituted position of

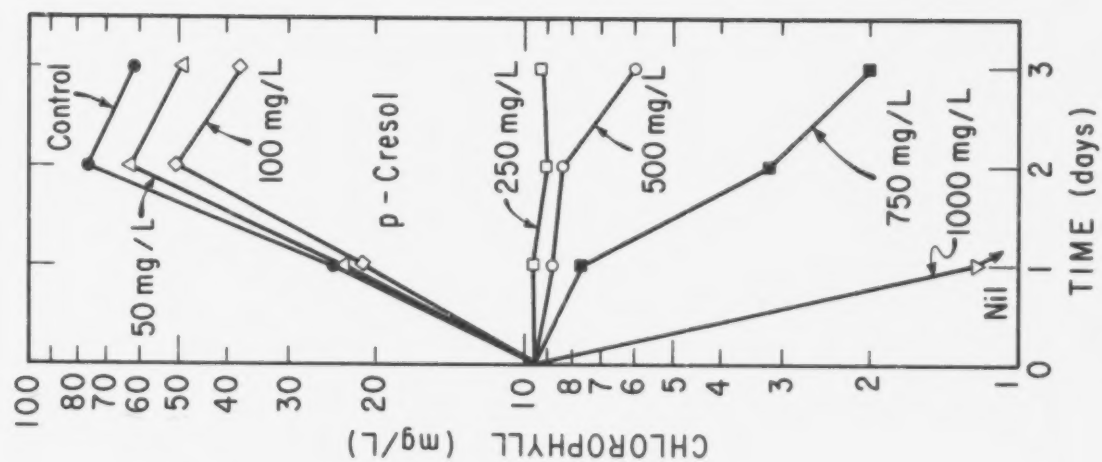


FIG. 3-21. EFFECTS OF p-CRESOL

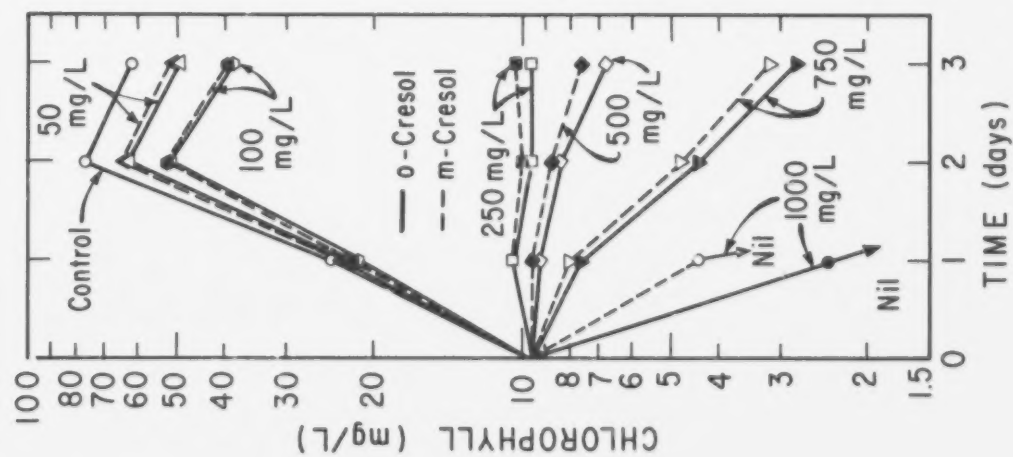


FIG. 3-20. EFFECTS OF o- AND m-CRESOLS

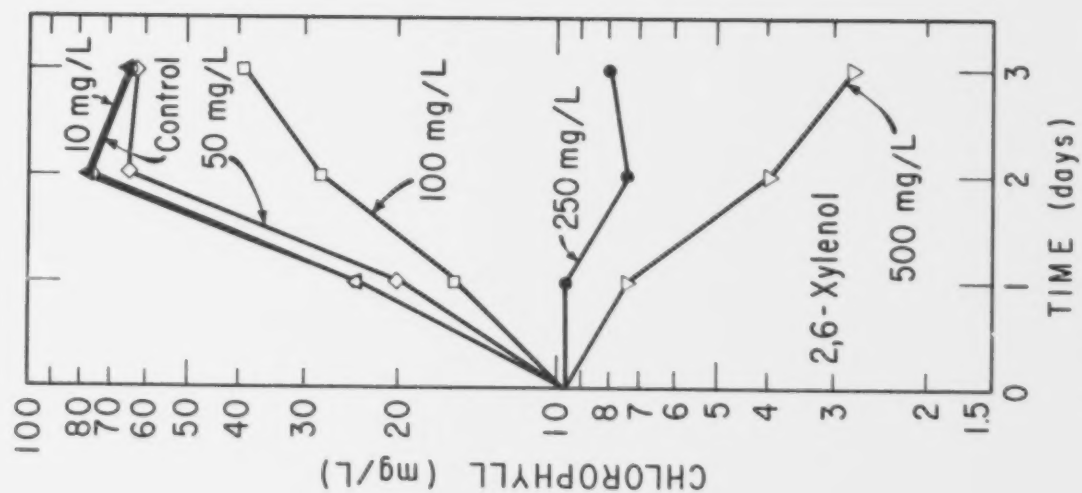


FIG. 3-23. EFFECTS OF
2,6 - XYLENOL

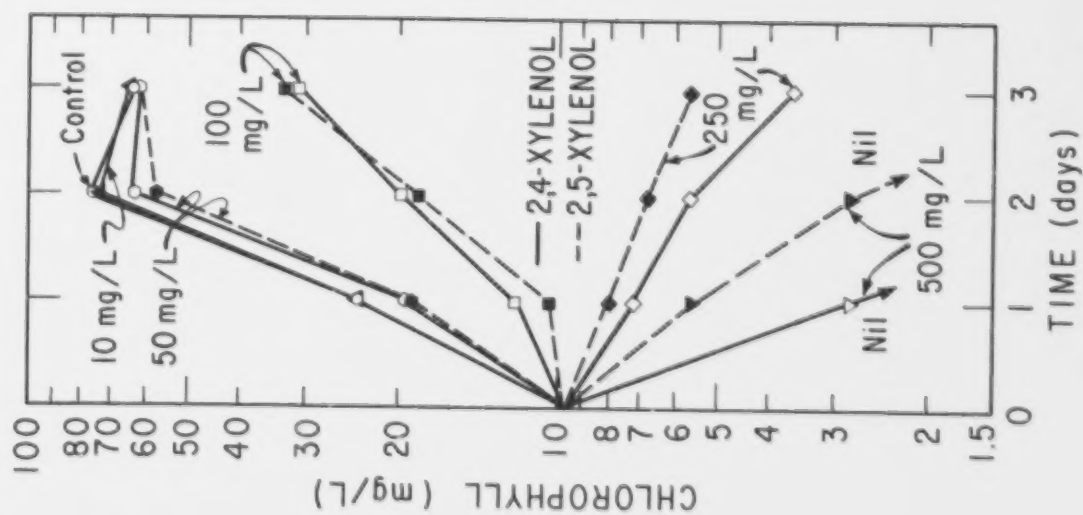


FIG. 3-22. EFFECTS OF
2,4-AND 2,5- XYLENOLS

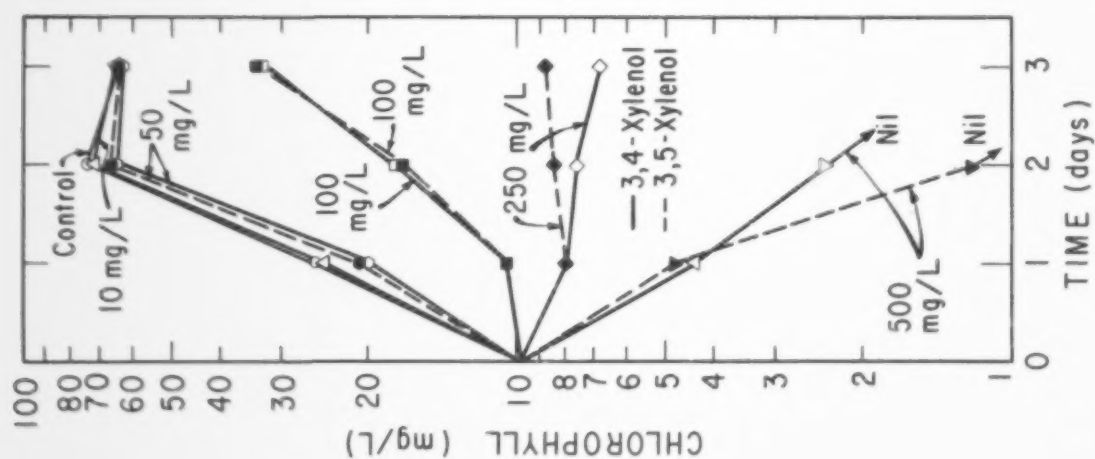


FIG. 3-24. EFFECTS OF 3,4- AND 3,5-XYLENOLS

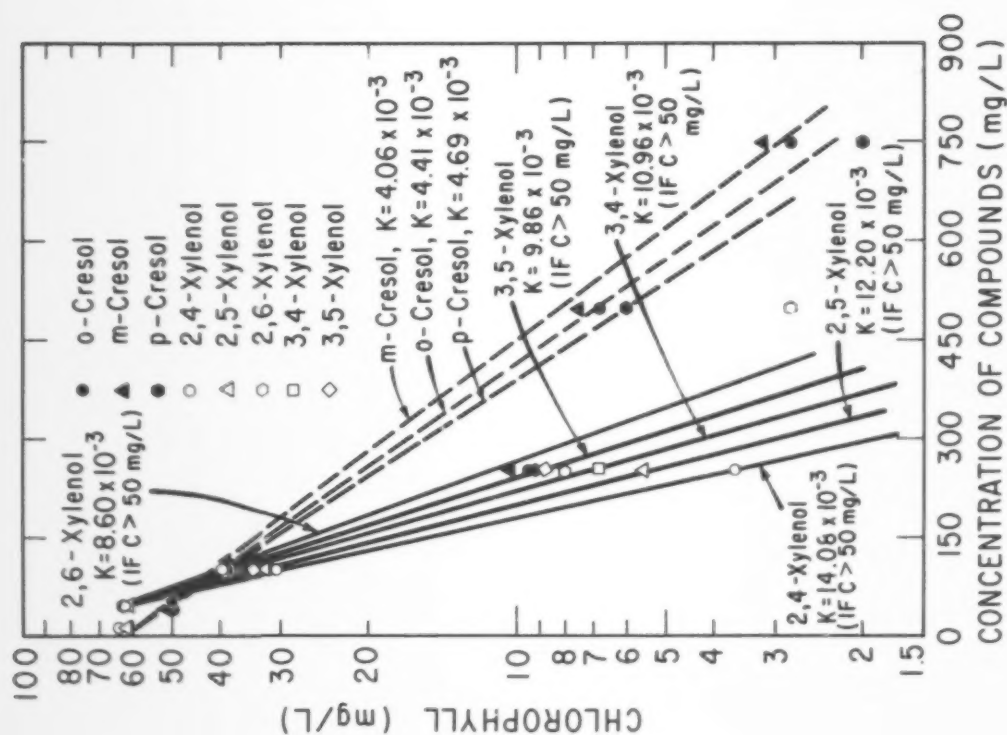


FIG. 3-25. TOXICITY CONSTANTS OF CRESOLS AND XYLENOLS

the alkyl group does not significantly affect the relative toxicity of the compound. These results are in good agreement with those reported by Fieser (17) as regard to the relative toxicity of alkyl-substituted phenols to bacteria. Fieser stated that, in general, the more alkyl groups introduced to the phenol nucleus, the higher the toxicity is. The position of the alkyl group is relatively unimportant, but the chain length is significant. Fieser gave the example that the effectiveness toward Gram-negative bacteria reached a peak with a straight-chain of five to six carbon atoms; beyond this length the potency decreased. The effectiveness toward Gram-positive bacteria also increased at least until the chain contains nine carbon atoms, and from this point on the substances are too insoluble to permit measurement.

Alkyl-substituted phenols possess the ability to decrease surface tension, and may probably owe at least some of their increased toxicity to this property, since many surface-active agents are potent bactericides.

In studying the bio-degradation of aromatic compound by phenol-adapted bacteria, Tabak, Chamber and Kabler (71) also found that the relative amounts of oxygen consumption for the three cresols were in the order of: *m*-cresol > *o*-cresol > *p*-cresol. Comparing this with the relative magnitudes of toxicity for the three cresols, it is obvious that the relative order of toxicity of cresols to Chlorella pyrenoidosa is consistent with the relative resistance of these compounds to bio-degradation.

Nitro-Substituted Phenols

Five nitro-substituted phenols were involved in this study; they were o-, m-, and p-nitrophenols, 2,4-dinitrophenol, and 4,6-dinitro-o-cresol. The results reflect that introduction of a nitro group to phenol greatly increases the toxicity to Chlorella pyrenoidosa. However, introduction of a second nitro group does not further increase the toxicity; instead, it decreases the toxicity in comparison with the mono-nitrophenols. The reason for this decrease in toxicity is unknown.

As shown in Figs. 3-26 and 3-27, there were a definite difference, though it was small, in the relative toxicity of the three isomers of nitrophenols. Para-nitrophenol was the most toxic, o-isomer the next and m-isomer the least. However, the patterns of variations of chlorophyll concentrations as a function of time were very similar, except different in the relative magnitudes, for the three nitrophenols. At a concentration of 1 mg/L all of the three nitrophenols exhibited no toxicity to Chlorella pyrenoidosa. At the concentration of 25 mg/L the chlorophyll synthesis was completely inhibited by p-nitrophenol. If the concentrations were above 50 mg/L, all of these three nitrophenols almost completely inhibited the chlorophyll synthesis.

It is very interesting to note that 2,4-dinitrophenol has a lower toxicity than the three mono-nitrophenols. As shown in Fig. 3-28, 2,4-dinitrophenol did not exhibit any slight toxic effects on Chlorella pyrenoidosa at the concentration of 10 mg/L; on the other hand, the rate of chlorophyll synthesis increased slightly. At the

Table 3-13. Test Data for Nitro-Substituted Phenols

Compound	Concentration of Compound (mg/L)	Chlorophyll (mg/L)			
		After 0 Day	After 1 Day	After 2 Days	After 3 Days
o-Nitrophenol	Control	9.6	24.4	77.0	62.0
	1	9.6	21.6	72.0	64.0
	10	9.6	14.0	44.0	32.0
	25	9.6	9.0	11.6	15.6
	35	9.6	8.0	6.8	6.8
	50	9.6	6.0	3.2	2.8
	100	9.6	4.4	0	0
m-Nitrophenol	Control	9.6	24.4	77.0	62.0
	1	9.6	24.0	70.6	60.0
	10	9.6	15.0	49.2	40.0
	25	9.6	12.0	15.6	19.2
	50	9.6	7.6	6.4	3.2
	100	9.6	4.0	3.2	0
p-Nitrophenol	Control	9.6	24.4	77.0	62.0
	1	9.6	25.6	78.8	62.0
	10	9.6	13.6	37.2	24.4
	25	9.6	10.0	9.6	8.8
	50	9.6	7.8	5.0	1.3
	100	9.6	3.7	0	0
2,4-Dinitro- phenol	Control	9.7	25.6	77.6	68.8
	1	9.7	24.0	75.6	63.2
	10	9.7	18.8	79.2	71.6
	25	9.7	16.0	25.0	28.0
	50	9.7	10.8	9.0	4.0
	100	9.7	9.2	6.4	2.2
4,6-Dinitro- o-cresol	500	9.7	4.0	0	0
	Control	9.7	25.6	77.6	68.8
	1	9.7	25.6	80.0	64.8
	10	9.7	21.6	80.4	67.2
	25	9.7	18.4	32.0	40.0
	50	9.7	9.4	8.0	4.8
	100	9.7	9.2	9.8	2.0
	500	9.7	7.6	3.6	0

concentration of 50 mg/L, 2,4-dinitrophenol was less effective in inhibiting the chlorophyll synthesis as compared with the three mono-nitrophenols.

The addition of a methyl group to 2,4-dinitrophenol did not alter substantially the toxicity to algae. The results of 2,4-dinitrophenol and 4,6-dinitro-o-cresol, or systematically called 2,4-dinitro-6-methylphenol, are shown in Table 3-13 and Fig. 3-28.

Price (62) also found that chlorophyll synthesis in Euglena gracilis was insensitive to dinitrophenol at low concentrations and the rate was actually increased about 30 percent when algal cells were suspended in 10^{-3} M phosphate buffer with a dinitrophenol concentration of 3×10^{-5} M, which is equivalent to 5.5 mg/L. In view of the finding that 2,4-dinitrophenol failed to inhibit the chlorophyll formation, Price concluded that oxidative phosphorylation could not be the energy source for this synthetic process. It might be that chlorophyll synthesis was dependent on photophosphorylation. Kessler (38) reported that at a concentration of 2×10^{-3} M with a pH of 6.5, 2,4-dinitrophenol inhibited the nitrite reduction in Ankistrodesmus braunii and Chlorella ellipsoidea.

Figure 3-29 shows the toxicity constants of the five nitro-substituted phenols. A threshold toxic concentration of 10 mg/L was established for 2,4-dinitrophenol and 4,6-dinitro-o-cresol in calculating the toxicity constants since these two compounds failed to show significant toxic effects at concentrations below 10 mg/L.

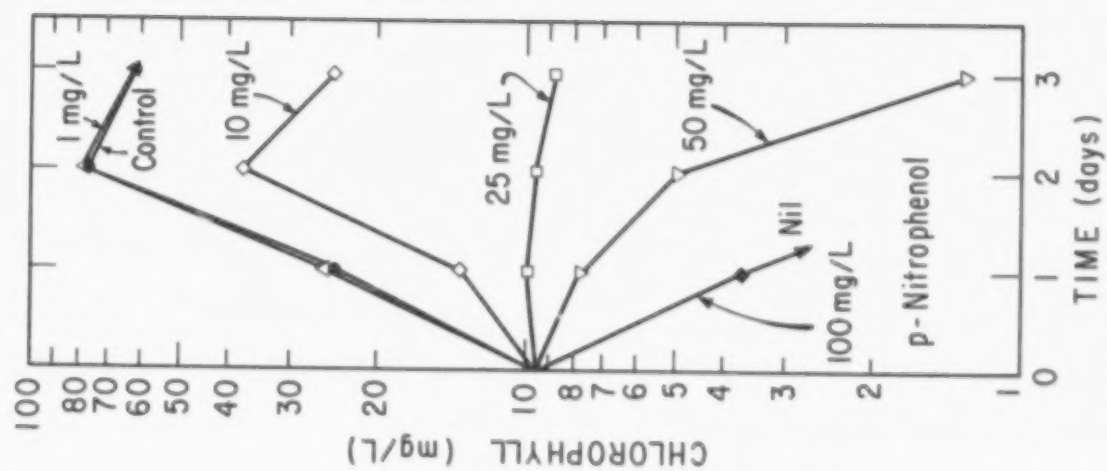


FIG. 3-27. EFFECTS OF
o-NITROPHENOL

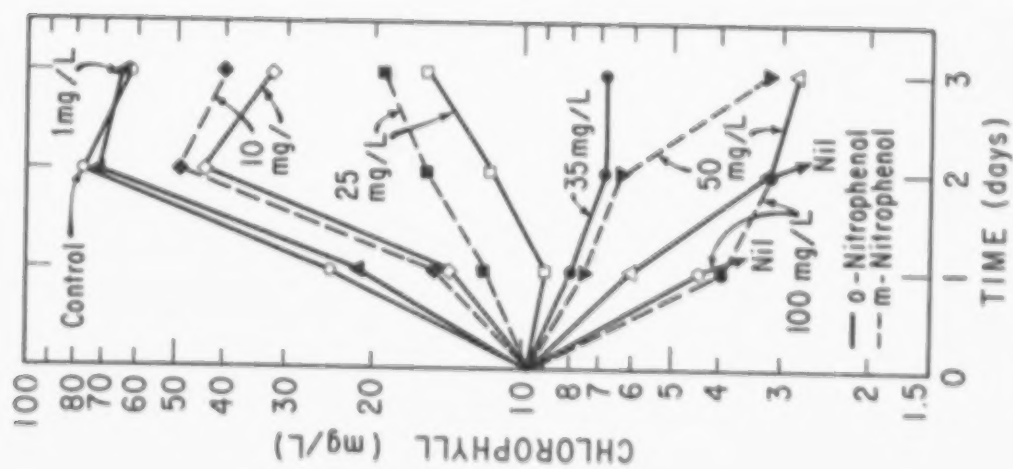


FIG. 3-26. EFFECTS OF
o- AND m-NITROPHENOLS

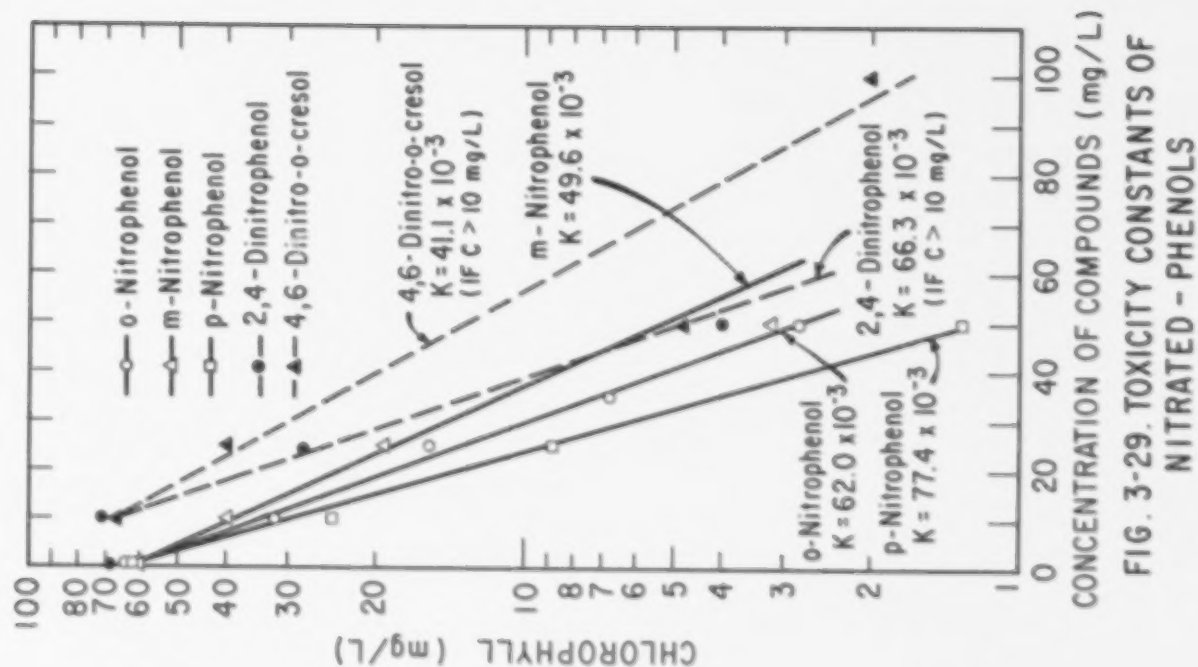


FIG. 3-29. TOXICITY CONSTANTS OF NITRATED - PHENOLS

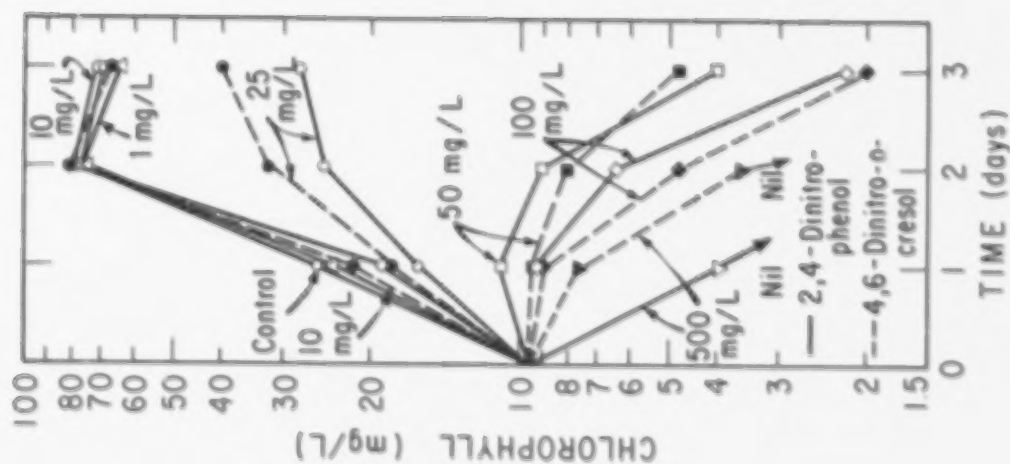


FIG. 3-28. EFFECTS OF 2,4-DINITROPHENOL AND 4,6-DINITRO-O-CRESOL

Amino-Substituted Phenols

Three mono-aminophenols and one aminophenolic-like compound, i.e., 2-amino-1-phenol-4-sulfonic acid, were investigated. These aminophenols are frequently found in some of the industrial wastes. For example, m-aminophenol is used in the manufacture of dyes and p-aminophenol is very important as a photographic developer.

The toxicity data showed that o-aminophenol had a higher toxicity than the other two isomers. The introduction of a sulfonic group to o-aminophenol drastically decreased the toxicity. Presumably that 2-amino-1-phenol-4-sulfonic acid behaved like a derivative of sulfonic phenol rather than a derivative of aminophenol. The test data are shown in Table 3-14 and Figs. 3-30 to 3-33.

As shown in Figs. 3-30 and 3-31, at the concentration of 100 mg/L m- and p-aminophenols inhibited the chlorophyll synthesis for the first day of incubation, and then the photosynthetic activity recovered rapidly. However, at the same concentration of 100 mg/L, o-aminophenol almost completely inhibited chlorophyll synthesis through the test period. At the concentration of 250 mg/L p-aminophenol showed complete inhibition of chlorophyll synthesis through the three-day test period while m-isomer still showed only an inhibition of one day.

Noticeably, the addition of a sulfonic group to o-aminophenol greatly decreased the toxicity to Chlorella pyrenoidosa. As shown in Fig. 3-31 the compound of 2-amino-1-phenol-4-sulfonic acid exhibited not a slightly toxic effect even at a concentration as high as 500 mg/L. At a higher concentration of 1000 mg/L this sulfonic phenol began to

inhibit chlorophyll synthesis for one day and then the photosynthetic process resumed during the next day and the chlorophyll content reached the same concentration of control at the conclusion of the test.

Table 3-14. Test Data for Amino-Substituted Phenols

Compound	Concentration of Compound (mg/L)	Chlorophyll (mg/L)			
		After 0 Day	After 1 Day	After 2 Days	After 3 Days
o-Aminophenol	Control	10.4	24.6	76.8	64.8
	10	10.4	12.8	44.4	58.4
	50	10.4	8.8	15.6	28.0
	100	10.4	3.6	7.6	7.6
	250	10.4	2.8	2.4	2.0
	500	10.4	2.8	0	0
m-Aminophenol	Control	10.4	24.6	76.8	64.8
	10	10.4	24.0	60.0	63.2
	50	10.4	18.4	44.4	58.0
	100	10.4	10.0	38.5	40.0
	250	10.4	9.6	14.8	21.6
	500	10.4	8.8	8.0	5.6
	1,000	10.4	6.4	4.4	0
p-Aminophenol	Control	10.4	24.6	76.8	64.8
	10	10.4	22.8	56.8	56.8
	50	10.4	16.8	40.0	52.4
	100	10.4	9.6	28.8	37.6
	250	10.4	5.6	4.4	4.4
	500	10.4	0	0	0
2-Amino-1-phenol-4-sulfonic acid	Control	10.4	24.6	76.8	64.8
	100	10.4	24.0	74.8	64.4
	500	10.4	24.0	73.6	61.6
	1,000	10.4	12.4	36.6	63.6
	1,500	10.4	10.4	21.6	28.0
	2,000	10.4	8.4	8.4	6.0

The toxicity constants of these four amino-substituted phenols are shown in Figs. 3-32 and 3-33. Since 2-amino-1-phenol-4-sulfonic acid did not show significant toxic effects at concentrations up to 1000 mg/L, a threshold toxic concentration of 1000 mg/L was used in the calculation of the toxicity constant.

It has been suggested that certain o-aminophenols are carcinogenic because o-aminophenols are potent chelating agents and have ability to coordinate with metals in the body.

According to Myers (56), limitations of growth in Chlorella pyrenoidosa are readily demonstrated when iron, manganese or zinc are omitted from the nutrient solution. Limitations of growth by omission of added boron, calcium, cobalt, copper or molybdenum do not appear significantly since all of these elements are present in sufficient quantities as impurities in the major salts. Price (62) also emphasized that in an iron limited medium, rate of chlorophyll formation in Euglena gracilis is directly linearly proportional to the iron concentration in cells. Therefore, in order to determine whether the higher toxicity of o-aminophenol than the other two isomers is owing to the chelation of metals in the culture medium, a test was conducted by using a culture medium which was supplemented with as much as five times of iron, zinc and manganese in the Knop's solution. The results reflected that the relative toxicity of o-aminophenol was not slightly altered by the supplementation of these metals to the medium. This suggested the conclusion that the higher toxicity of o-aminophenol to Chlorella pyrenoidosa was unlikely due to the formation of ligands of this

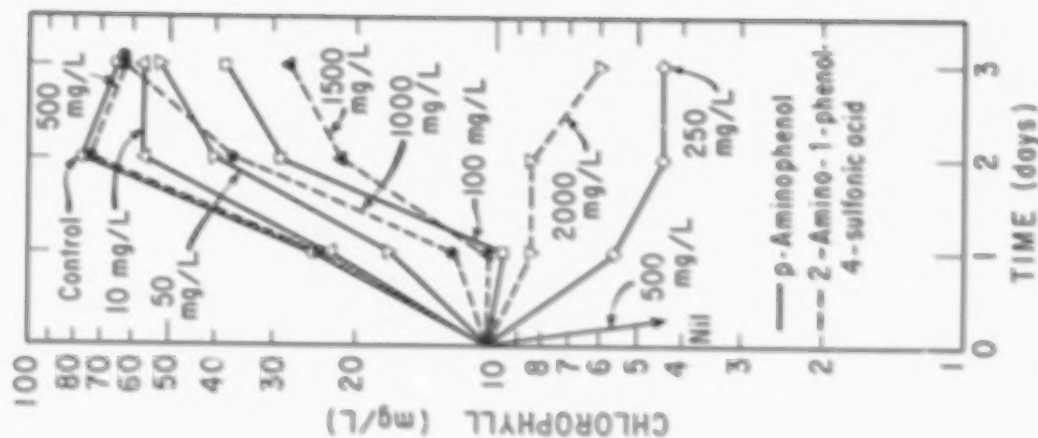


FIG. 3-31. EFFECTS OF p-AMINOPHENOL AND 2-AMINO-1-PHENOL-4-SULFONIC ACID

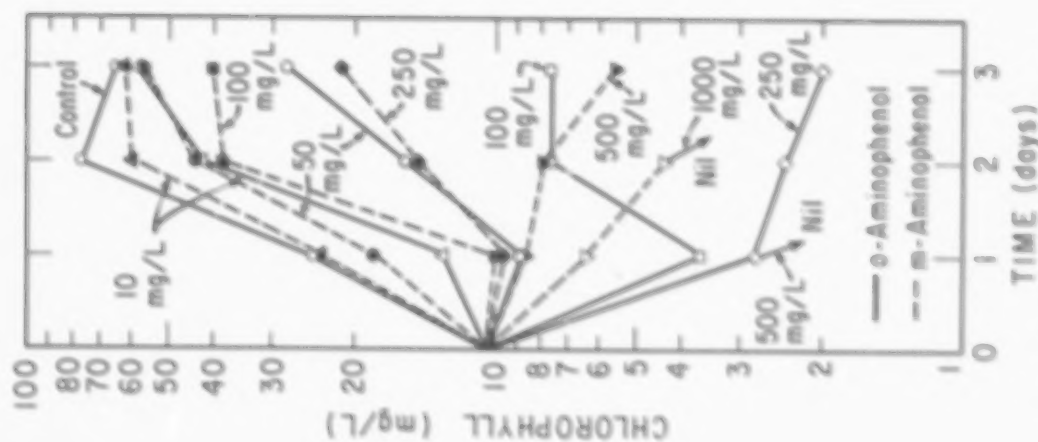


FIG. 3-30. EFFECTS OF o- AND m-AMINOPHENOL

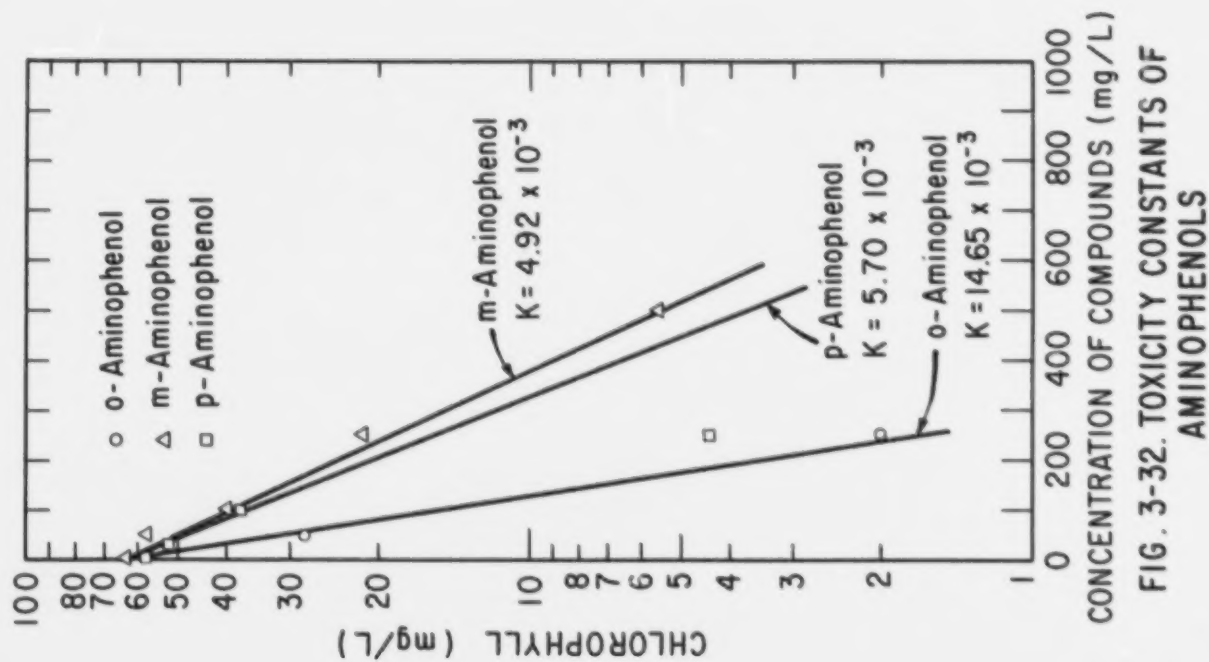


FIG. 3-32. TOXICITY CONSTANTS OF AMINOPHENOLS

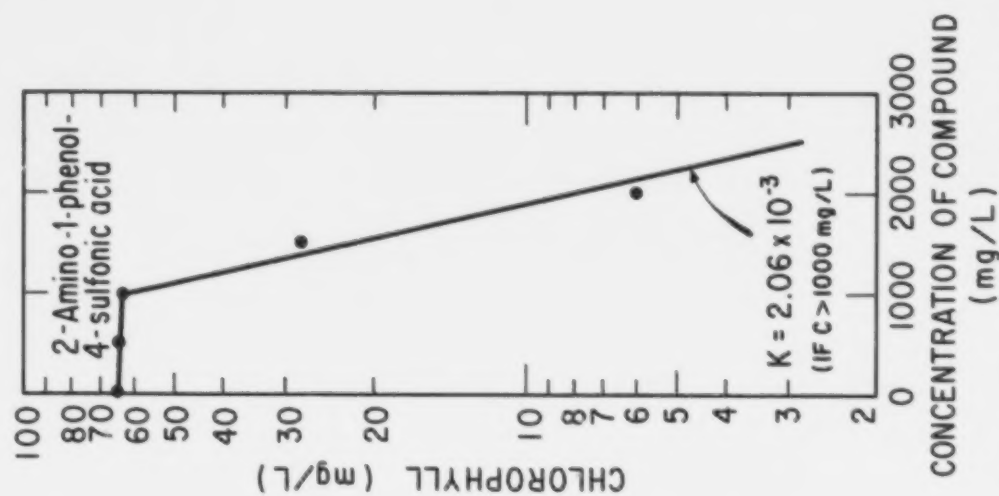


FIG. 3-33. TOXICITY CONSTANT OF 2-AMINO-1-PHENOL-4-SULFONIC ACID

compound with metals.

On the other hand, in correlating the chemical structure of the quinone-type compounds and the toxicity to blue-green algae, Fitzgerald (22) pointed out that any compound with two H atoms which can be oxidized from -OH or -NH group which are ortho or para to each other will give rise to a fairly stable oxidant and thus, in general, will exhibit higher toxic properties than meta-isomer. This explanation probably gives a good account for the fact that o- and p-aminophenols have higher toxicity than m-isomer to Chlorella.

Dihydric and Trihydric Phenols

Three dihydric and two trihydric phenols were selected in this toxicity study. Three dihydric phenols included catechol, resorcinol and hydroquinone. Catechol is sometimes referred to as benzene-1,2-diol, o-dihydroxybenzene or pyrocatechol, while hydroquinone is sometimes referred to as quinol. Two trihydric phenols selected were pyrogallol, or known as pyrogalllic acid, and phloroglucinol. Sanitary engineers often use alkaline solutions of pyrogallol to remove oxygen from gases. Pyrogallol is also a minor constituent of spent tan liquors. When these liquors are discharged to streams containing iron, inky black ferric pyrogallate is formed. Catechol and hydroquinone are reducing agents and hence are used as a photographic developer while resorcinol is not so powerful a reducing agent as the o- and p-isomers. The relative magnitudes of toxicity of these three dihydric phenols to Chlorella pyrenoidosa are

in general consistent with their strengths of reducing powers.

As shown in Figs. 3-34 and 3-35, at the concentration of 100 mg/L resorcinol did not exhibit significant toxicity to Chlorella pyrenoidosa, while catechol and hydroquinone inhibited the chlorophyll synthesis for one day. At a concentration of 500 mg/L resorcinol did not show much influence on chlorophyll synthesis as the chlorophyll content increased steadily and reached a value of 43.2 mg/L after three days of contact. On the other hand, at the same concentration of 500 mg/L catechol inhibited the process for the first two days and hydroquinone almost completely inhibited the process; the resulted chlorophyll concentrations at the end of the test for catechol and hydroquinone, respectively, were 15.6 and 6.0 mg/L.

It is very interesting to note that the relative magnitudes of toxicity of catechol and resorcinol to Chlorella pyrenoidosa are less than that of phenol. Hydroquinone has only a slightly higher toxicity than phenol. Fieser (17) also reported that polyhydric phenols are generally less potent bactericides than phenol.

It has been shown (75) that both -OH group in catechol can lose a proton to give a doubly negatively charged chelate group which will form complexes with elements having a high affinity for oxygen, such as potassium and molybdenum. However, it seems unlikely that the toxicity of catechol to algae is owing to the chelate-forming reaction with metals, since hydroquinone, which is a relative non-chelating compound, has a higher toxicity than catechol. Tabak, Chambers, and Kabler (71) found that hydroquinone was more resistant to bio-degradation than catechol and resorcinol. Therefore, the

relative toxicity of dihydric phenols is also a function of their bio-degradability. Also, the stable oxidant formed from p- and o-isomers of dihydric phenols also partly account for the characteristics of higher toxicity than m-isomer.

Table 3-15. Test Data for Dihydric and Trihydric Phenols

Compound	Concentration of Compound (mg/L)	Chlorophyll (mg/L)			
		After 0 Day	After 1 Day	After 2 Days	After 3 Days
Catechol (Benzene-1,2- diol, o-Dihy- droxybenzene, or Pyrocatechol)	Control	9.7	30.8	65.6	61.6
	50	9.7	18.8	58.0	56.0
	100	9.7	9.2	24.8	35.2
	500	9.7	7.6	9.2	15.6
	1,000	9.7	6.8	5.8	5.3
	2,000	9.7	5.8	5.2	2.8
	3,000	9.7	5.6	2.4	0
Resorcinol	Control	9.7	30.8	65.6	61.6
	50	9.7	29.4	56.8	55.6
	100	9.7	28.4	52.4	49.6
	500	9.7	21.8	33.6	43.2
	1,000	9.7	20.0	16.0	18.0
	2,000	9.7	8.8	7.8	5.2
	3,000	9.7	6.4	4.2	0
Hydroquinone (Quinol)	Control	9.7	30.8	65.6	61.6
	50	9.7	15.8	52.4	51.2
	100	9.7	7.8	15.2	24.4
	500	9.7	6.0	7.6	6.0
	1,000	9.7	4.4	4.4	3.2
	2,000	9.7	4.4	3.0	1.6
	3,000	9.7	4.0	0	0
Pyrogallol (Pyrogallie acid)	Control	10.4	26.4	79.2	68.4
	10	10.4	24.0	76.8	70.4
	100	10.4	7.6	9.6	9.6
	250	10.4	7.3	9.6	9.6
	500	10.4	6.8	9.2	9.6
	1,000	10.4	5.6	4.4	9.6
Phloroglucinol	Control	10.4	26.4	79.2	68.4
	100	10.4	24.8	74.4	67.2
	250	10.4	22.8	72.8	64.8
	500	10.4	20.0	63.2	64.8
	1,000	10.4	18.4	62.8	63.2

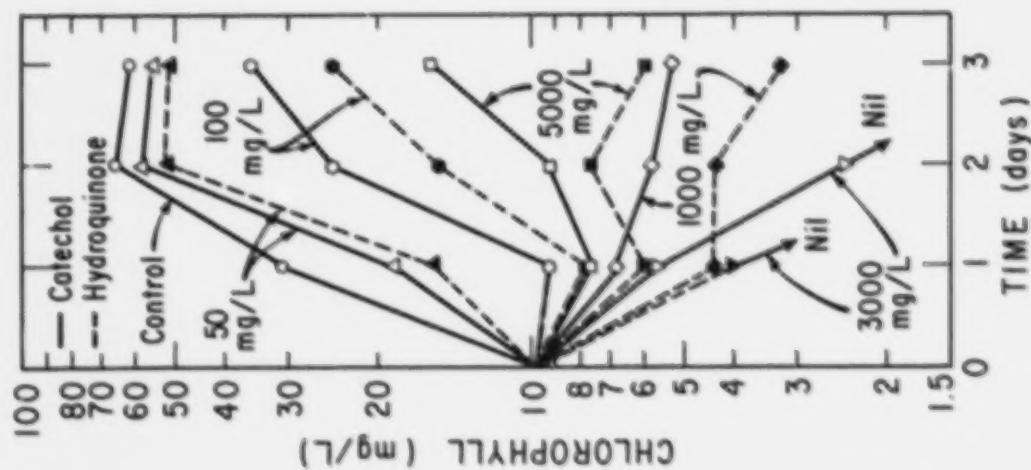


FIG. 3-34. EFFECTS OF CATECHOL AND HYDROQUINONE

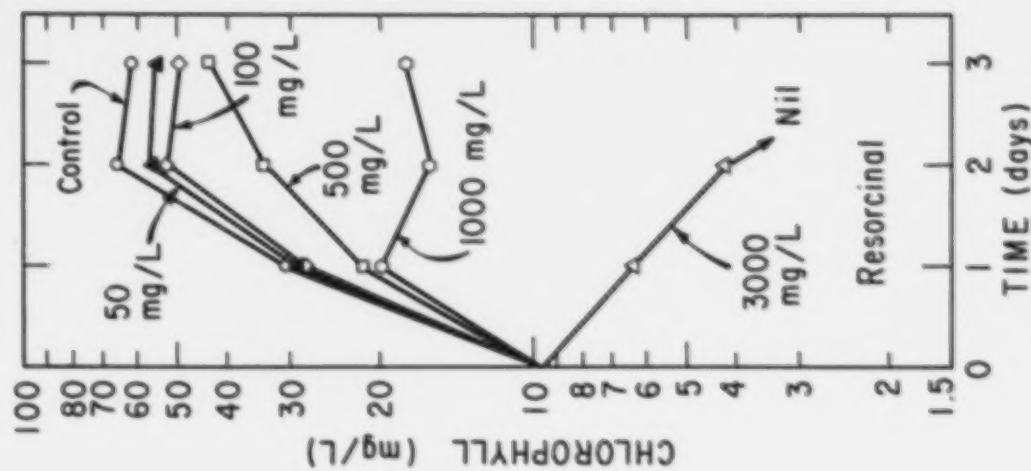


FIG. 3-35. EFFECTS OF RESORCINOL

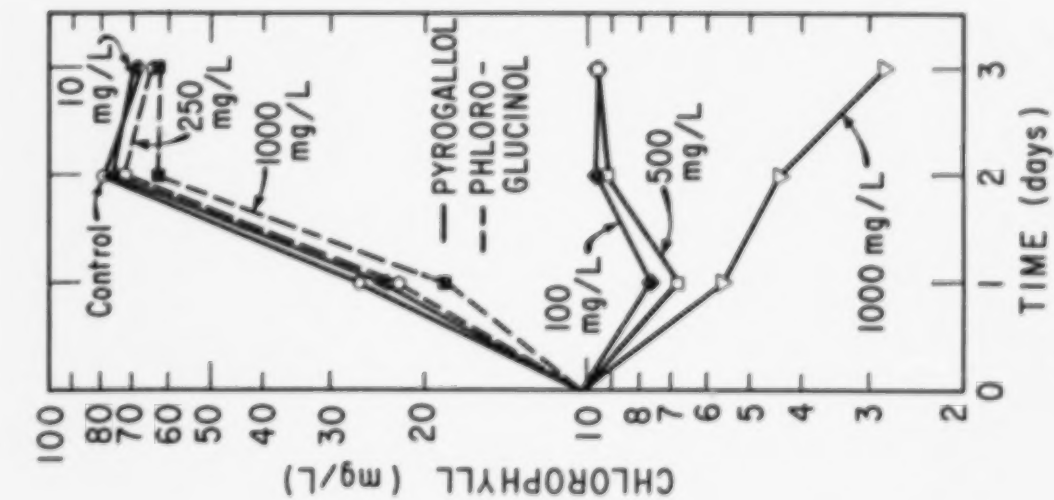


FIG. 3-36. EFFECTS OF
PYROGALLOL AND
PHLOROGLUCINOL

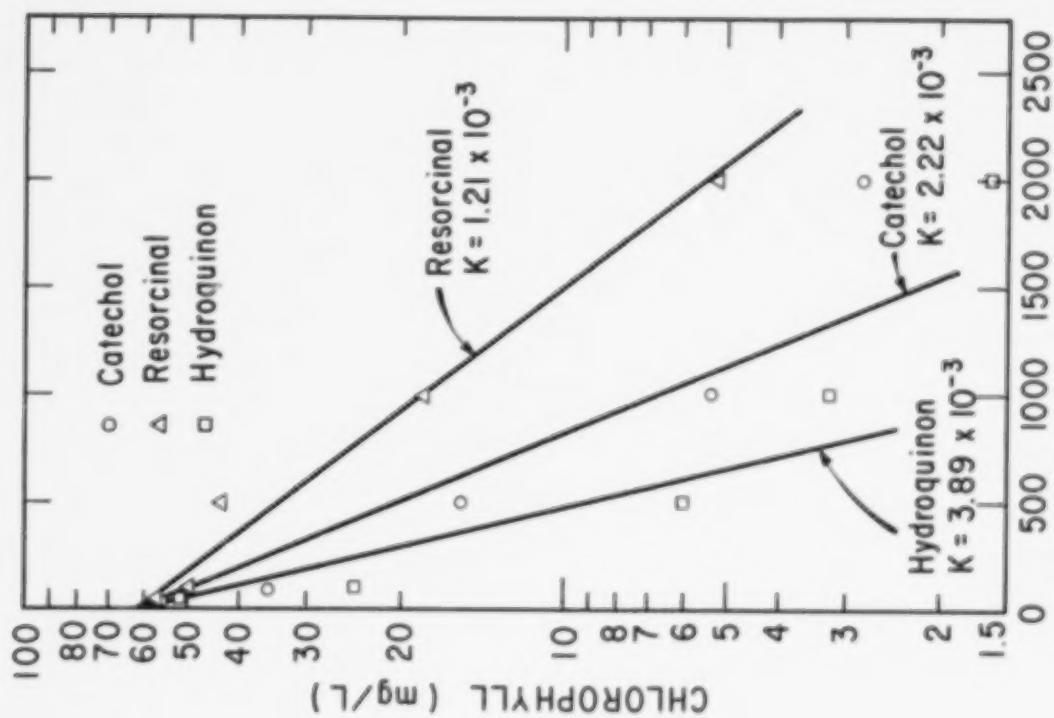


FIG. 3-37. TOXICITY CONSTANTS OF
DIHYDRIC PHENOLS

The two trihydric phenols exhibited great difference in toxicities to Chlorella pyrenoidosa, as shown in Fig. 3-36. Pyrogallol inhibited the chlorophyll synthesis at a concentration of 100 mg/L, while phloroglucinol exhibited no significant toxicity to algae even at a concentration as high as 1000 mg/L. The great difference of the toxicity data between these two trihydric phenols might be due to the combined effects of: a.) pyrogallol is a stronger reducing agent than phloroglucinol and hence has a higher toxicity; b.) pyrogallol formed a very dark-brown solution upon dissolving in the culture medium at concentrations greater than 100 mg/L and thereby blocked the incidence of light which is indispensable for photosynthesis.

The toxicity constants of the three dihydric phenols are shown in Fig. 3-37. In case of these two trihydric phenols, the chlorophyll reductions are not logarithmic to the increase of concentration of the compounds. Therefore, there is no way to calculate such toxicity constants for these two trihydric phenols.

Pesticides

The widespread use of toxic chemicals, as a means of pest control or weed control, arouses interest in the effects of these agents on all plants and animals and other microorganisms. Since the introduction of DDT as an insecticide, other highly toxic organic compounds have been synthesized and used in large quantities over land and aquatic areas. However, the toxic effects of these organic pesticides on algae have not been established yet.

Two insecticides and six herbicides were selected in this investigation. The insecticides selected were DDT and lindane while the herbicides were 2,4-D, sodium salt of 2,4-D, 2,4,5-T, Dow sodium TCA, Dow Estaron 99 and Dow Formula 40. The last three herbicides are the products of Dow Chemical Company. The relative toxicity of these eight pesticides are shown in Table 3-16 and Figs. 3-38 through 3-46.

The old name for DDT is dichlorodiphenyl trichloroethane. Now the commonly accepted name is 1,1,1-trichloro-2,2-di-(p-chlorophenyl)-ethane. DDT has been one of the most prominent insecticides and indeed, it is lethal to a wider range of insects than any other insecticides now known. The toxic effects may be due to combination of the action of the chlorobenzene units, which cause respiratory poison, with that of chloroform, which is lipid-soluble narcotic (17).

The results shown in Fig. 3-38 indicated that DDT was not toxic to Chlorella pyrenoidosa even at a saturated solution. Since the solubility of DDT is only about 0.2 mg/L (74), the non-toxic nature of DDT may partly be owing to its very low solubility.

Similar to this finding, Ukeles (74) also reported that DDT colloidal solution at a concentration of 0.6 mg/L did not affect the growth of a Chlorella species while Palmer and Maloney (47) found that DDT was not toxic to six species of freshwater algae at 2 mg/L. However, Thirumurthi (73) found that 25 percent DDT in xylene solvent at 125 mg/L eliminated the chlorophyll in Chlorella pyrenoidosa after three days of contact. Since xylene is highly toxic to this alga (39), the elimination of chlorophyll was believed due to the toxic effects of xylene rather than DDT itself.

Table 3-16. Test Data for Pesticides

Compound	Concentration of Compound (mg/L)	Chlorophyll (mg/L)			
		After 0 Day	After 1 Day	After 2 Days	After 3 Days
DDT	Control	9.2	26.2	76.4	68.8
	10% saturation	9.2	25.4	74.2	68.0
	25% saturation	9.2	25.4	72.2	68.0
	50% saturation	9.2	23.6	70.4	62.8
	100% saturation	9.2	23.4	68.4	64.0
Lindane	Control	9.2	26.2	76.4	68.8
	1.0	9.2	22.0	55.4	58.4
	2.5	9.2	18.4	32.4	40.0
	5.0	9.2	14.2	18.0	20.0
	7.0	9.2	10.4	8.2	8.0
	8.5	9.2	6.6	4.2	3.2
	10.0	9.2	5.4	2.8	0
2,4-D	Control	9.2	26.0	74.8	67.4
	5	9.2	28.4	77.6	70.0
	50	9.2	22.1	58.8	55.2
	100	9.2	17.5	37.8	42.0
	220	9.2	12.1	17.5	20.0
	440	9.2	10.0	9.0	8.6
Sodium salt of 2,4-D	Control	9.2	26.0	74.8	67.4
	100	9.2	16.2	34.8	38.8
	500	9.2	7.6	7.8	5.6
	1,000	9.2	6.0	6.4	4.0
	5,000	9.2	3.2	3.2	0
2,4,5-T	Control	9.0	25.2	74.8	64.8
	20	9.0	24.4	72.6	63.2
	50	9.0	22.0	58.0	58.4
	100	9.0	14.2	32.1	37.6
	196	9.0	8.4	14.4	20.0
Sodium TCA*	Control	8.6	23.1	71.0	64.8
	500	8.6	21.2	62.0	61.2
	1,000	8.6	20.8	54.2	57.2
	5,000	8.6	18.1	51.2	54.0
	10,000	8.6	15.3	46.4	49.6
	30,000	8.6	9.2	10.8	11.2
	50,000	8.6	2.4	2.8	2.8

Table 3-16. Test Data for Pesticides (Continued)

Compound	Concentration of Compound (mg/L)	Chlorophyll (mg/L)			
		After 0 Day	After 1 Day	After 2 Days	After 3 Days
Esteron 99*	Control	9.6	25.2	74.2	66.0
	100	9.6	23.6	60.4	55.6
	500	9.6	20.2	51.2	49.6
	1,000	9.6	16.8	29.6	37.2
	5,000	9.6	10.4	11.6	12.8
	10,000	9.6	7.8	6.8	2.4
Formula 40*	Control	9.6	25.2	74.2	66.0
	100	9.6	23.8	67.4	64.0
	500	9.6	21.6	57.2	58.4
	1,000	9.6	20.8	42.0	44.6
	5,000	9.6	13.8	19.4	18.4
	10,000	9.6	8.4	8.1	6.4

* Products of Dow Chemical Company.

Lindane is a γ -isomer of hexachlorocyclohexane (HHC) and also one of the most commonly used insecticides. This insecticide was very toxic to Chlorella pyrenoidosa, as shown in Fig. 3-38. At a saturated solution, i.e., 10 mg/L, lindane completely eliminated the chlorophyll at the end of the test. At a lower concentration, the reduction of chlorophyll was logarithmic to the increase of lindane concentration, as shown in Fig. 3-39. Lindane has a toxicity constant of 29×10^{-3} , which is approximately 100 times as toxic as phenol.

Ukeles (74) also found that lindane was relatively toxic to a Chlorella species and at the concentration of 7.5 mg/L reduced the growth rate to a value of only 36 percent of the control.

It was reported that in an aqueous solution containing 1 mg/L of lindane, fish suffered more than bacteria. The bacterial

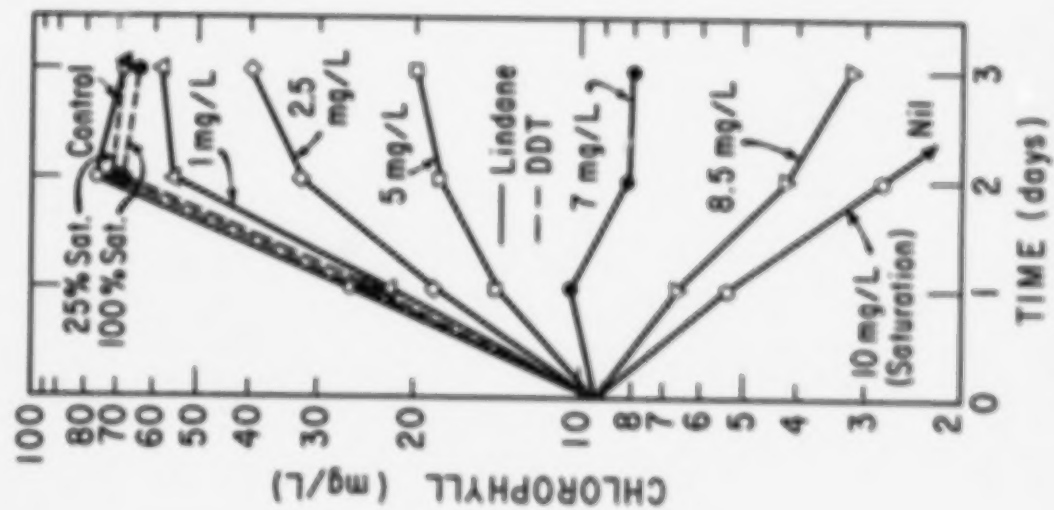


FIG. 3-38. EFFECTS OF DDT AND LINDANE

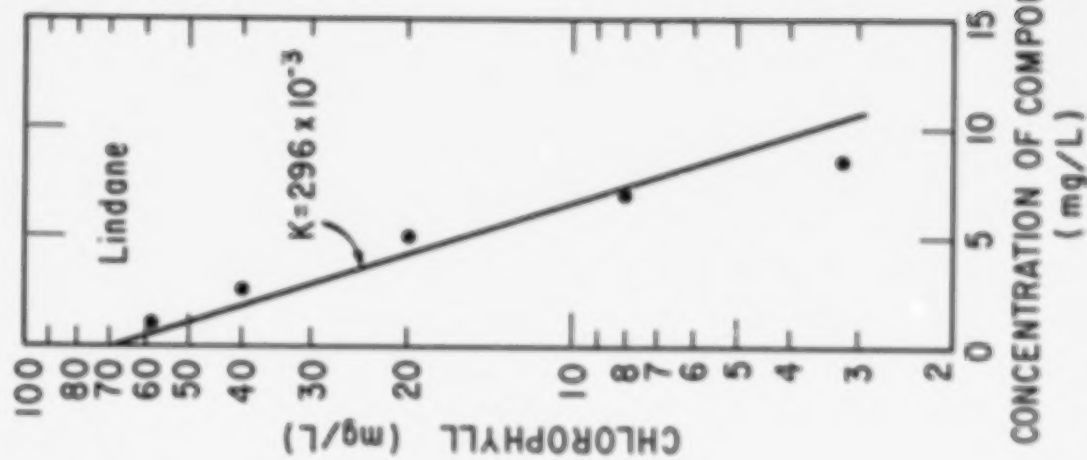


FIG. 3-39. TOXICITY CONSTANT OF LINDANE

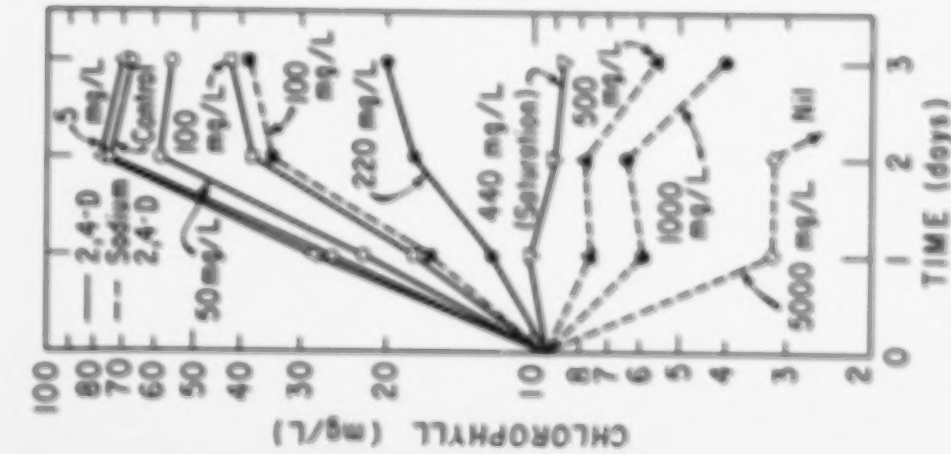


FIG. 3-40. EFFECTS OF 2,4-D AND SODIUM SALT OF 2,4-D

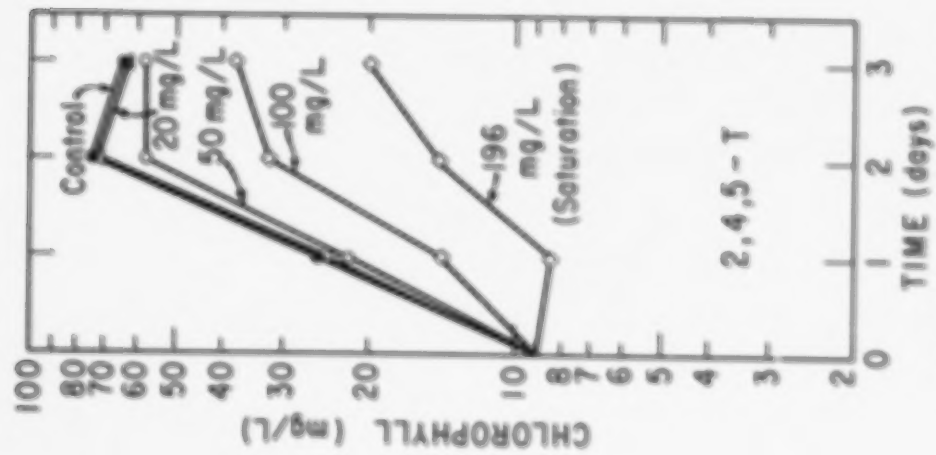


FIG. 3-41. EFFECTS OF 2,4,5-T

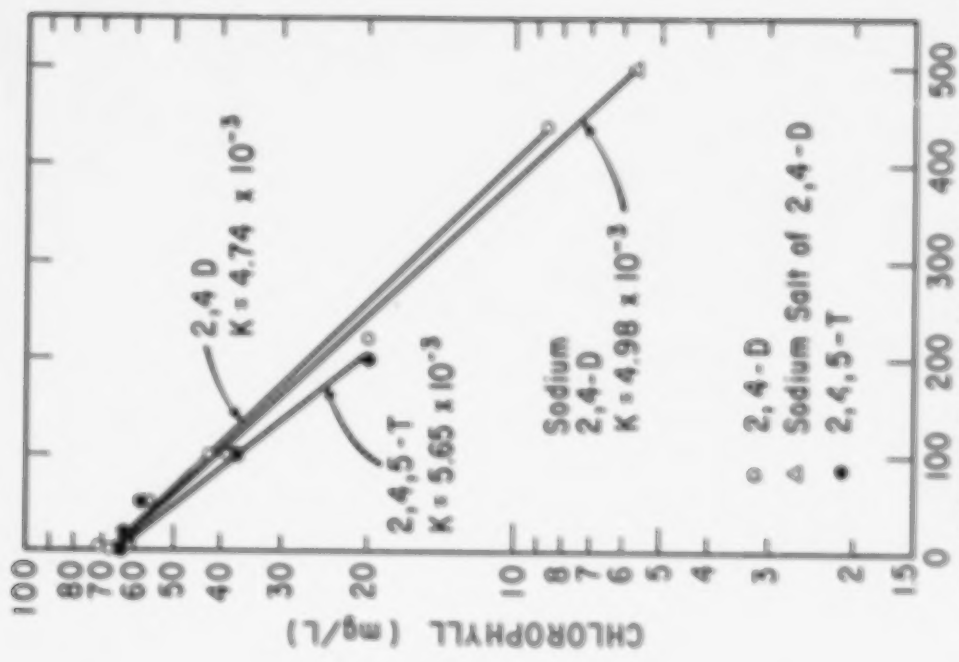


FIG. 4-42. TOXICITY CONSTANTS OF 2,4-D, SODIUM 2,4-D AND 2,4,5-T

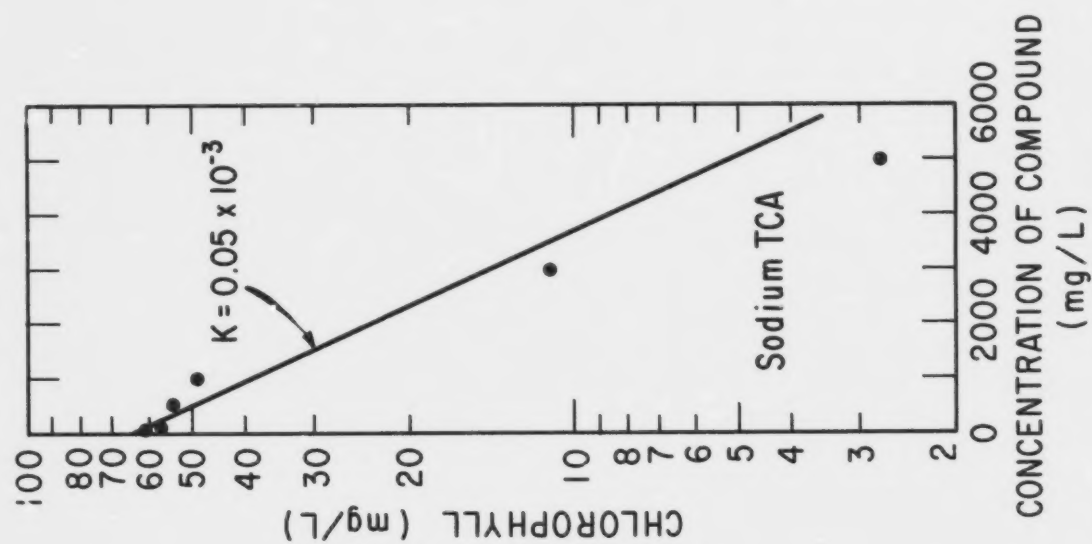


FIG. 3-44. TOXICITY CONSTANT OF DOW SODIUM TCA

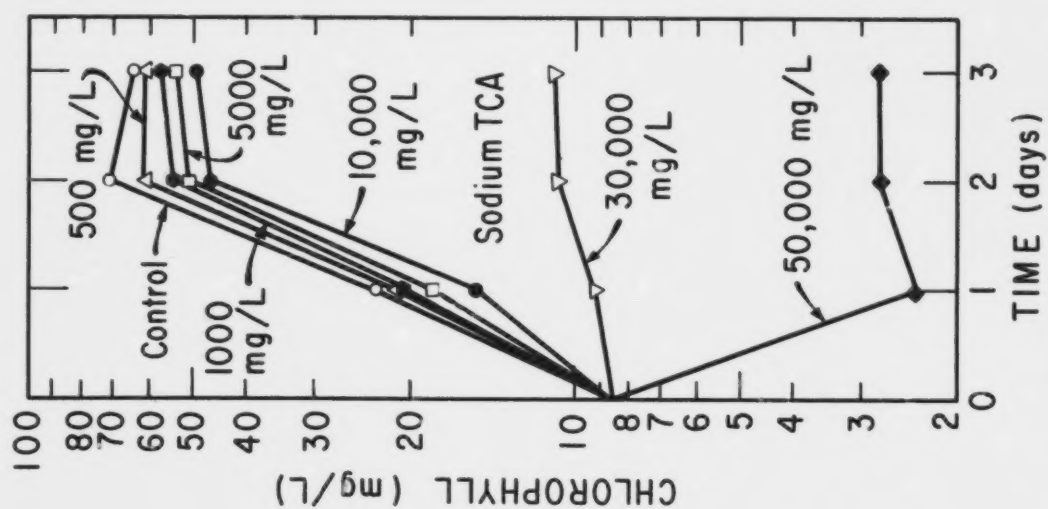


FIG. 3-43. EFFECTS OF DOW SODIUM TCA

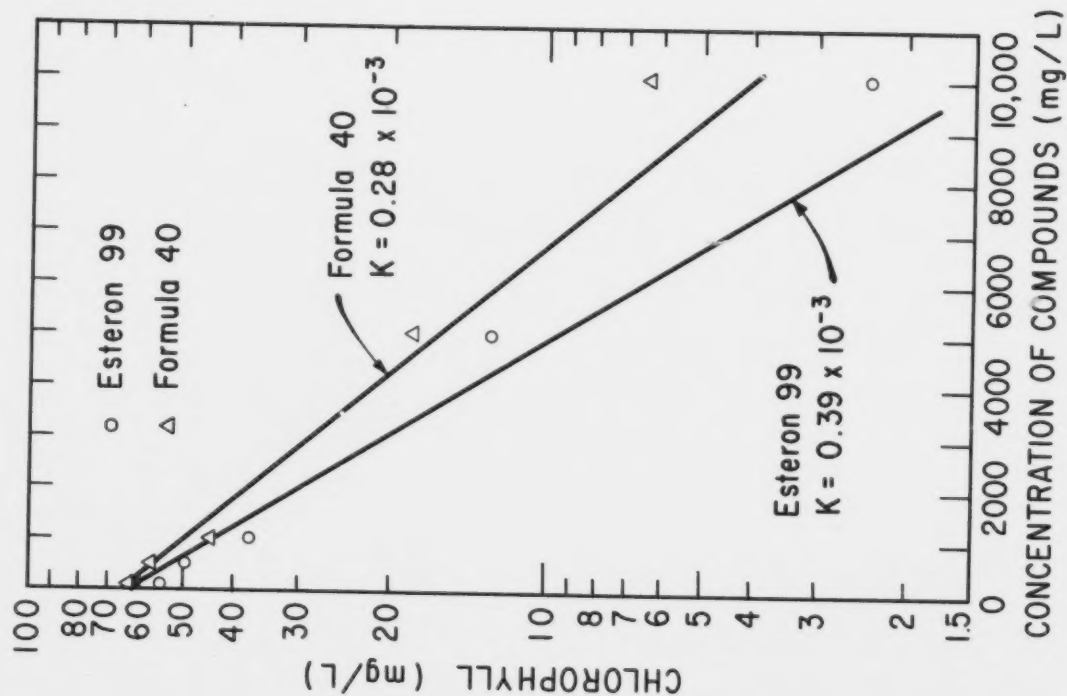


FIG. 3-46. TOXICITY CONSTANTS OF
DOW ESTERON 99 AND
DOW FORMULA 40

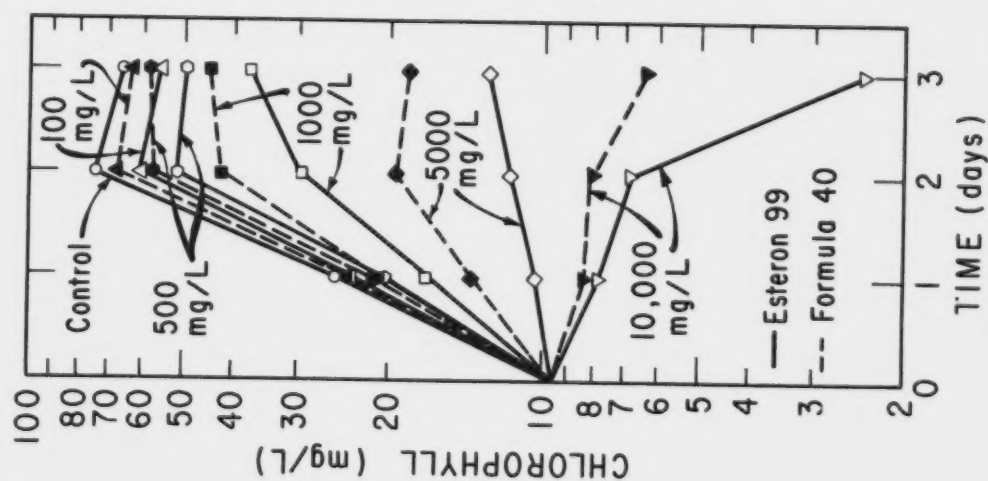


FIG. 3-45. EFFECTS OF
DOW ESTERON 99 AND
DOW FORMULA 40

content decreased during the first 24 hours and then, as a result of adaptation, increased to former values. Most of protozoa and algae were killed at this concentration.

It is of interesting to note that some of the most toxic weed killers used on higher plants were without appreciable effects on Chlorella pyrenoidosa. 2,4-Dichlorophenoxyacetic acid (2,4-D) is a plant growth hormone and can stimulate various physiological processes at the low concentration. However, at a higher concentration, 2,4-D possesses the herbicidal property against most leafed-weeds, although it is relatively non-toxic to monocotyledonous plants. The results shown in Fig. 3-40 indicated that 2,4-D at a low concentration of 5 mg/L seemed to stimulate slightly the chlorophyll synthesis, though the increase of the rate of chlorophyll synthesis was not appreciable. At a higher concentration, 2,4-D became an inhibitor to algae. The rates of chlorophyll formation were only about 60 percent and 30 percent, respectively, of the control at the concentration of 100 mg/L and 220 mg/L of 2,4-D. At the concentration of saturation, i.e., 440 mg/L, 2,4-D completely inhibited the synthesis of chlorophyll through the test period.

The sodium salt of 2,4-D exhibited very similar toxic effect as 2,4-D. Sodium salt of 2,4-D is soluble in water. At the concentration of 5000 mg/L it eliminated the chlorophyll at the end of the test.

Mashtakov (49) found that sodium salt of 2,4-D at the concentration of 100 mg/L inhibited the photochemical activity of chloroplasts in corn plants having 3 to 4 leaves. However,

Fitzgerald (22) reported that several species of blue-green algae appeared to develop normally in the presence of 2,4-D at 250 mg/L.

The compound of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) exhibited only a slightly higher toxicity than 2,4-D, as shown in Fig. 3-41. However, at a lower concentration of 20 mg/L, 2,4,5-T did not exhibit significant toxic effects. This finding was in good agreement with that reported by Fitzgerald (22). The solubility of 2,4,5-T is 196 mg/L, at which level approximately 60 percent of the activity of chlorophyll synthesis was inhibited.

The toxicity constants of 2,4-D, sodium salt of 2,4-D and 2,4,5-T are shown in Fig. 3-42. These three compounds are approximately two times as toxic as phenol to Chlorella pyrenoidosa.

According to the manufacturer's label, sodium TCA contains 95 percent of active ingredient, sodium trichloroacetate, and 5 percent of inert ingredients. It is used for the control of grass seedlings and certain established perennial grasses such as Johnsongrass. The data shown in Fig. 3-43 reflected that sodium TCA was relatively non-toxic to algae. At the concentration of 10,000 mg/L, TCA still showed no appreciable effects on the chlorophyll synthesis. The toxicity constant shown in Fig. 3-44 revealed that sodium TCA has only a toxicity as low as one-sixtieth of phenol.

Esteron 99 contains 41.0 percent of the propylene glycol butyl ether esters of 2,4-D and 59.0 percent of inert ingredients, while Formula 40 contains 65.8 percent of the alkanolamine salts (of the ethanol and isopropanol series) of 2,4-D and 34.2 percent of inert ingredients. Both of these compounds are soluble in water

and are used for the selective control of many broad leafed-weeds in non-crop areas. However, these two powerful weed killers did not exhibit high toxicity to Chlorella pyrenoidosa, as shown in Fig. 3-45. At a concentration as high as 5000 mg/L these two herbicides inhibited about 60 to 70 percent of chlorophyll synthesis activity. Formula 40 had a slightly lower toxicity than Esteron 99 to algae. The toxicity constants shown in Fig. 3-46 indicates that Esteron 99 and Formula are about one tenth as toxic as phenol to Chlorella pyrenoidosa.

Toxicity of the Combination of Two Organic Compounds

The majority of the organic compounds investigated in this study exhibits a toxicity in the manner of the first-order reaction, that is, the toxic effects of the suppression of chlorophyll formation is logarithmic to the increase of the concentration of the compound. Therefore, the fraction of chlorophyll remaining, as compared with the control, in an algal suspension containing a toxic compound can be predicted according to the following relationship:

$$\text{Fraction of chlorophyll remaining} = \frac{Q}{Q_0} = e^{-K \cdot C} \quad \dots \text{Eq. 3-2}$$

where Q_0 = chlorophyll content in the control which has no toxic compound,
 Q = chlorophyll content in the presence of the toxic compound,
 K = toxicity constant of the toxic compound,
 C = concentration of the toxic compound.

It might be assumed that the toxicity of the combination of two single compounds is probably additive, if there is no chemical

interreaction occurred between these two chemicals. For example, phenol at 100 mg/L and p-bromophenol at 50 mg/L inhibit the chlorophyll synthesis to only, respectively, 66.5 percent and 58 percent of the control; now, if 100 mg/L of phenol and 50 mg/L of p-bromophenol are present together in an algal suspension, the fraction of chlorophyll remaining might be assumed to be $66.5\% \times 58\% = 38.6\%$. If this assumption is true, Eq. 3-2 can thus be expressed in the following general form:

$$\text{Fraction of chlorophyll remaining} = e^{-\sum_{i=1}^I K_i \cdot C_i} \quad \text{.....Eq. 3-3}$$

where K_i and C_i are, respectively, the toxicity constant and the concentration of the i^{th} toxic compound.

As a step toward evaluating the validity of Eq. 3-3, a test was conducted by adding two toxic compounds together to algal suspensions. The test data are shown in Table 3-17.

A comparison is shown in Fig. 3-47 between the predicted fraction of chlorophyll remaining, calculated by the multiplication of the fraction of each single compound, and the actual fraction of chlorophyll remaining obtained from the experimental data. From this figure it is evident that the experimental data are very close to the predicted values. Therefore, the generality of Eq. 3-3 seems to be valid, at least as far as the phenolic-type compounds are concerned.

Table 3-17. Toxicity of the Combination of Two Compounds

Compound and Its Concentration	Fraction of Chlorophyll Remaining (%)		
	After 1 Day	After 2 Days	After 3 Days
Phenol 100 mg/L	69.0	45.0	66.5
p-Bromophenol 50 mg/L	69.0	39.0	58.0
Phenol 100 mg/L plus p-bromo- phenol 50 mg/L	50.4	21.0	30.0
o-Chlorophenol 50 mg/L	76.0	55.6	85.0
Phenol 100 mg/L plus o-chloro- phenol 50 mg/L	55.0	26.2	50.2
p-Nitrophenol 10 mg/L	56.0	55.5	42.5
Phenol 100 mg/L plus p-nitrophenol 10 mg/L	45.0	32.0	38.0
p-Cresol 100 mg/L	78.8	69.5	61.3
Phenol 100 mg/L plus p-cresol 100 mg/L	62.0	42.2	48.0
2,4-Xylenol 100 mg/L	54.0	30.0	52.8
Phenol 100 mg/L plus 2,4-xylenol 100 mg/L	42.5	22.6	32.8
m-Aminophenol 100 mg/L	45.5	37.2	58.8
Phenol 100 mg/L plus m-aminophenol 100 mg/L	27.2	15.0	41.5
Hydroquinone 100 mg/L	33.4	23.9	42.5
Phenol 100 mg/L plus hydroquinone 100 mg/L	27.2	11.7	30.3

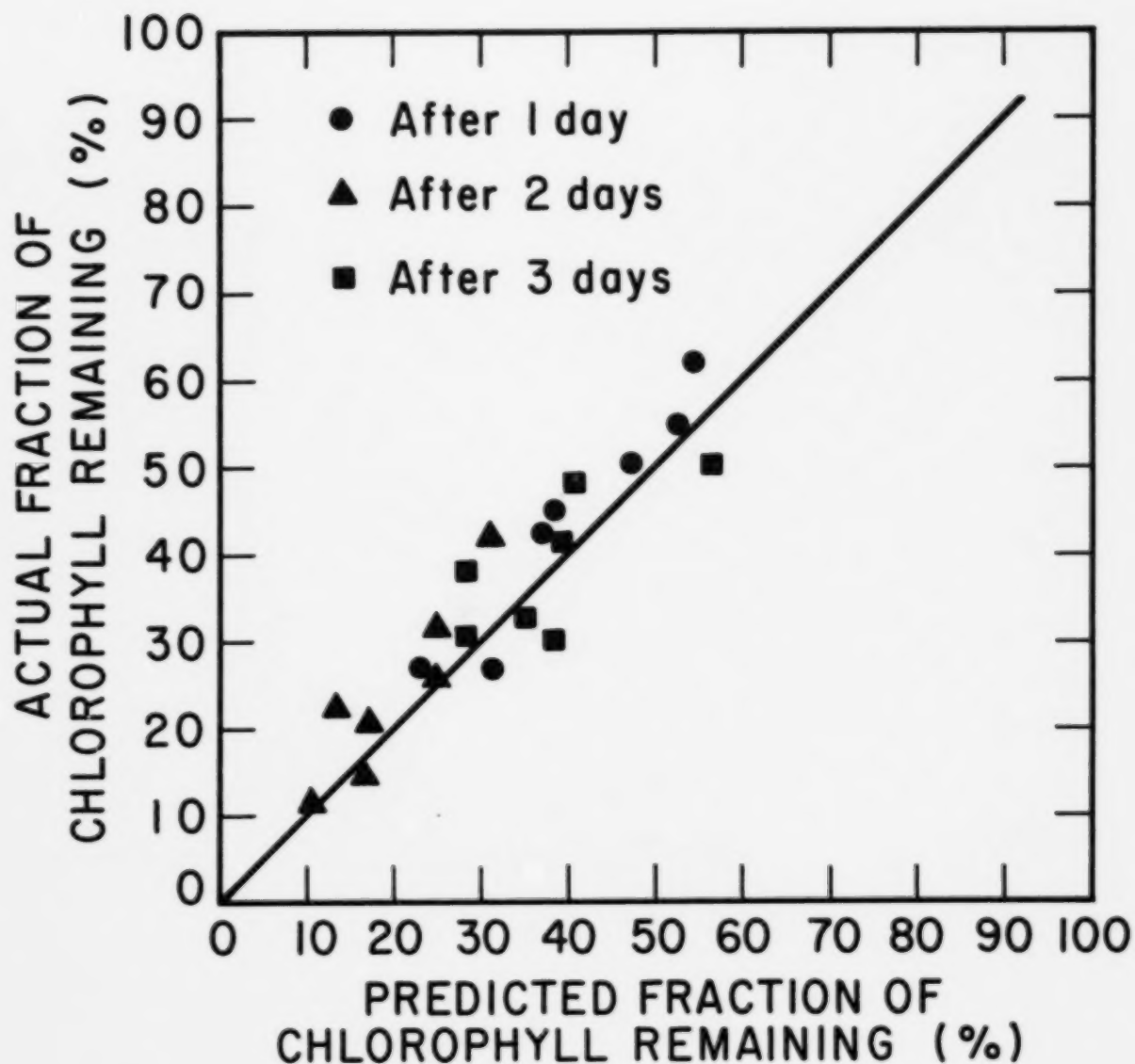


FIG. 3-47. TOXICITY OF THE COMBINATION OF TWO COMPOUNDS

CHAPTER IV

SUPPRESSION OF PHOTOSYNTHETIC GAS EXCHANGE

This experiment required an illuminated Warburg apparatus. A generally accepted manometric technique for the measurement of photosynthetic gas exchange was employed.

Experimental Equipment

A Precision Scientific Company Warburg Apparatus was used for the manometric measurement of photosynthetic oxygen evolution. Illumination was provided from ten 20-watt fluorescent lamps with attached reflectors. The lamps were suspended 24 cm above the water bath of the Warburg apparatus. A light-meter survey indicated that this arrangement of illumination facility provided approximately an intensity of 300 foot-candles at the location where the reaction vessels were positioned.

The conventional respiration flask, which has a center well, a side arm and a total volume of approximately 20 ml, was used as the reaction vessel. The volumes of these flasks together with the manometers were calibrated with the mercury method (75). Conventional Brodie solution was used as the manometer fluid (75). The water bath was controlled at a temperature of 25°C. A fan was used to blow over the surface of the water bath to prevent the accumulation of hot air in the vicinity of the manometer. Reaction flasks were shaken at a rate of 98 strokes per minute. The pH Meter and Centrifuge used in this experiment were the same as those described in Chapter III.

Measurement of Photosynthesis

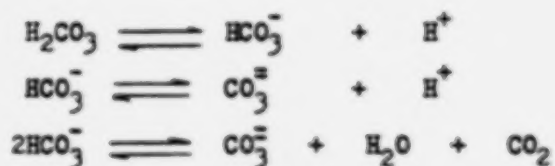
Several manometric techniques have been described by Myers (56). This experiment employed the direct method for the measurement of photosynthetic oxygen emission. A carbonate-bicarbonate buffer solution is used as the suspending fluid, and thus the carbon dioxide in both the liquid and gas phases is buffered. Therefore, photosynthetic oxygen becomes the only gas that exchanged with the gas phase in the reaction vessel.

The Warburg carbonate-bicarbonate buffer system, which has a total molarity of 0.2 M and a bicarbonate/carbonate ratio of 95/5, was used as the suspending fluid. The characteristics of this buffer solution are shown in Table 4-1.

Table 4-1. Characteristics of the Suspending Fluid

$[\text{HCO}_3^-]$ (M)	$[\text{CO}_3^{2-}]$ (M)	Total Molarity (M)	pH	$[\text{CO}_2]$ in Liquid ($\times 10^{-6}\text{M}$)	CO_2 in Gas (%)
0.190	0.010	0.200	8.79	635	1.89

In this carbonate-bicarbonate buffer solution, the following reactions



provide a buffer system for both carbon dioxide and hydrogen ion. It has been reported (56) that the carbon dioxide concentration in this solution is so well stabilized that errors less than one percent can be expected.

Emerson and Green (14) found that in a carbonate-bicarbonate mixture with a total molarity of 0.1 M and a pH of 9.0 or less, the concentration of free carbon dioxide is sufficient to "saturate" the maximum rate of algal photosynthesis. Therefore, the suspending fluid used in this experiment could provide enough free carbon dioxide for the maximum rate of algal photosynthesis.

Test Procedure

The algal culture used in this experiment was the same as that used in the previously reported test-tube study. In each test, 50 ml of algal suspensions were centrifuged, washed with the suspending fluid and centrifuged again. The supernatant was decanted. Algal cells were then resuspended in the suspending fluid with a final total volume of 25 ml to make a doubly concentrated algal cell suspension.

One ml of suspending fluid with predetermined amount of tested organic was added to each flask which was followed by the inoculation of another one ml of the concentrated algal cell suspension. The flask prepared in this manner, therefore, had a total fluid volume of 2 ml and a dry weight of 2 mg, which was equivalent to a packed cell volume of 7.6 cmm or a chlorophyll content of 0.076 mg.

Triplicated flasks were prepared for each concentration level of the tested compound. As soon as the algal suspension was inoculated, the flasks and manometer assembly were placed in the Warburg apparatus. A period of two hours was allowed for the adjustment of algal cells before the manometric pressure readings were taken.

Oxygen evolution was measured by monitoring pressure changes for a test period of two hours. The calculation of the flask constant as well as the conversion of the manometric pressure readings to the volumetric gas exchanges have been well described elsewhere (75).

Evaluation of Results

The compounds selected in this study included phenol, five chlorine-substituted phenols and four nitro-substituted phenols. The chlorinated phenols selected were the three isomers of chlorophenols, 2,4-dichlorophenol and 2,4,6-trichlorophenol. The nitrated phenols selected were the three isomers of nitrophenols and 2,4-dinitrophenol. The test data are shown in Tables 4-2 and 4-3, also in Figs. 4-1 through 4-10.

It must first be emphasized that the results obtained in this study represent only the suppression of photosynthesis during the specific short test period, i.e., from the second to the fourth hour after algal cells were exposed to the tested compounds. However, it seems reasonable to assume that as long as the algal cells were exposed to the toxic organic compound, there would be a continuous change in both biological and biochemical activities within the cells. Therefore, the rate of photosynthetic oxygen evolution is actually a function of time.

Furthermore, it must also be noted that in this experiment the illumination provided only an intensity of 300 foot-candles, which could not maintain a light-saturated photosynthesis.

The main purpose of this experiment was to demonstrate the fact that the photosynthetic oxygenation was actually suppressed when certain toxic organics were present. The extent of suppression was dependent upon the specific compound and its concentration level.

Table 4-2. Inhibitory Effects of Phenol and Chlorinated Phenols

Compound	Concentration of Compound (mg/L)	Photosynthesis (μ l O ₂ Production)				Percentage of Control (%)
		After 15 Min.	After 30 Min.	After 60 Min.	After 120 Min.	
Phenol	Control	12.0	24.0	48.4	96.0	
	50	11.3	22.6	44.8	86.8	90.5
	200	10.0	20.0	38.8	74.4	77.5
	500	8.6	17.0	35.4	71.0	74.0
	1,000	4.5	9.0	18.0	35.2	36.7
o-Chloro-phenol	Control	12.8	25.0	50.9	102.2	
	100	11.8	23.6	46.4	90.8	88.0
	250	10.1	19.9	39.6	78.8	77.0
	500	9.1	18.0	36.6	75.8	74.0
	1,000	5.5	11.0	22.2	40.0	39.0
m-Chloro-phenol	Control	11.4	22.8	45.5	89.0	
	100	10.1	20.2	38.6	72.7	81.7
	250	6.0	11.8	21.2	40.6	45.6
	500	-1.7	-3.2	-4.7	-6.3	-7.1
p-Chloro-phenol	Control	12.2	24.0	48.4	96.0	
	100	11.0	22.2	42.8	80.4	83.6
	250	7.2	15.2	28.4	58.8	61.3
	500	4.2	8.0	14.6	26.4	27.5
2,4-Dichloro-phenol	Control	13.8	27.7	55.5	110.0	
	10	12.5	25.0	48.4	98.0	89.2
	25	10.0	20.0	38.6	74.0	67.3
	50	8.6	17.4	32.0	62.0	56.4
	100	6.5	12.0	22.8	42.0	41.8
2,4,6-Tri-chlorophenol	Control	12.9	25.6	53.2	105.2	
	2.5	12.1	24.4	48.4	96.2	91.4
	5.0	11.5	23.0	46.0	90.4	85.8
	10.0	9.9	20.0	38.0	74.8	71.1
	50.0	7.4	15.2	29.6	56.0	53.2

Table 4-3. Inhibitory Effects of Nitrated Phenols

Compound	Concentration of Compound (mg/L)	Photosynthesis (nl O ₂ Production)				Percentage of Control (%)
		After 15 Min.	After 30 Min.	After 60 Min.	After 120 Min.	
o-Nitrophenol	Control	13.4	26.8	53.5	107.0	
	10	11.3	22.6	44.8	89.8	84.0
	25	10.2	20.3	40.6	81.6	76.3
	50	10.0	19.7	39.5	78.8	73.6
	100	8.9	18.0	37.0	73.6	69.0
m-Nitrophenol	Control	14.6	29.0	57.0	115.0	
	10	13.8	27.6	53.5	107.0	93.0
	50	11.8	23.7	45.6	91.2	79.4
	100	9.9	19.7	40.0	80.0	69.5
	500	-1.7	-2.6	-3.8	-5.6	-5.3
p-Nitrophenol	Control	14.6	29.2	55.6	110.0	
	10	12.6	25.0	48.5	90.0	81.8
	50	10.8	21.4	42.4	83.0	75.5
	100	9.2	18.3	36.2	72.0	65.5
	250	8.7	17.2	32.6	64.6	58.7
2,4-Dinitrophenol	Control	13.8	27.4	54.0	108.0	
	10	13.8	27.2	53.2	106.8	98.7
	50	12.0	23.8	45.6	88.8	82.2
	100	9.8	19.2	37.2	72.8	67.5

As shown in these data, the rates of photosynthetic oxygen evolution were inhibited, depending on the relative toxicity of the compound as well as the concentration. For example, the rates of oxygen emission were only 74.0 and 36.7 percent, respectively, of the control when the phenol concentrations were 500 and 1000 mg/L. At these two concentration levels, the chlorophyll contents at the end of the first day, as shown in Fig 3-5, were only about 35.3 percent ($= \frac{9.6}{27.2}$) and 26.8 percent ($= \frac{7.3}{27.2}$), respectively, of the control. Apparently, as long as the algal cells were exposed to the phenol solution, there was a continuously increasing suppression of their photosynthetic activities.

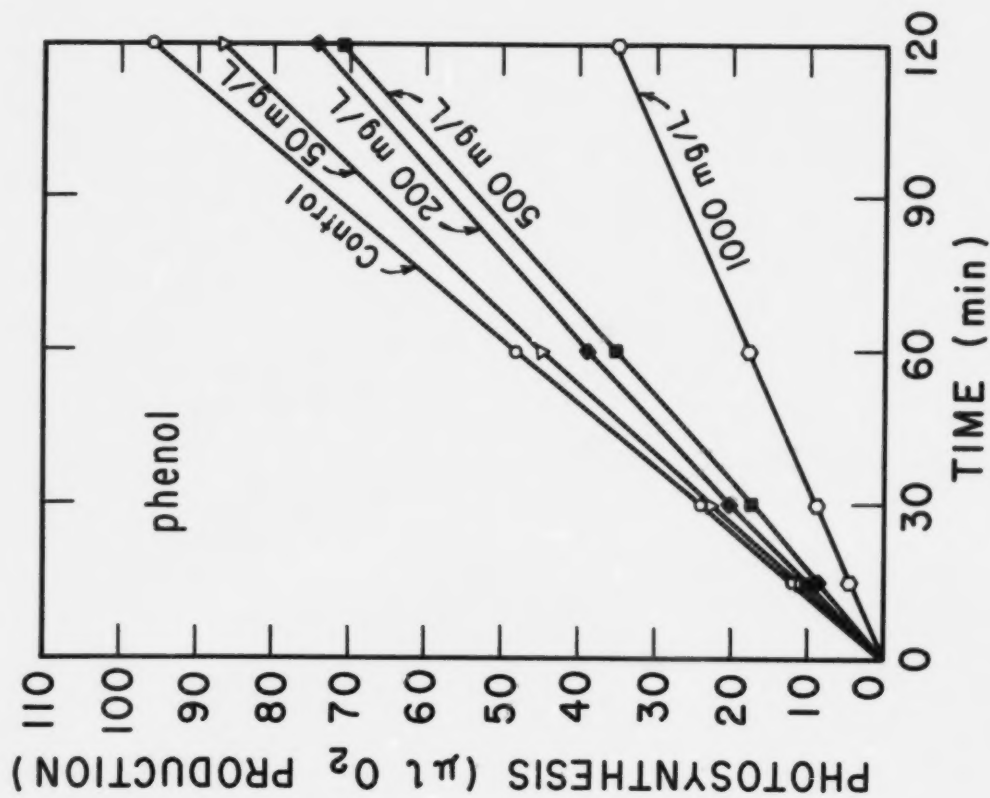


FIG. 4-1. INHIBITORY EFFECTS
OF PHENOL

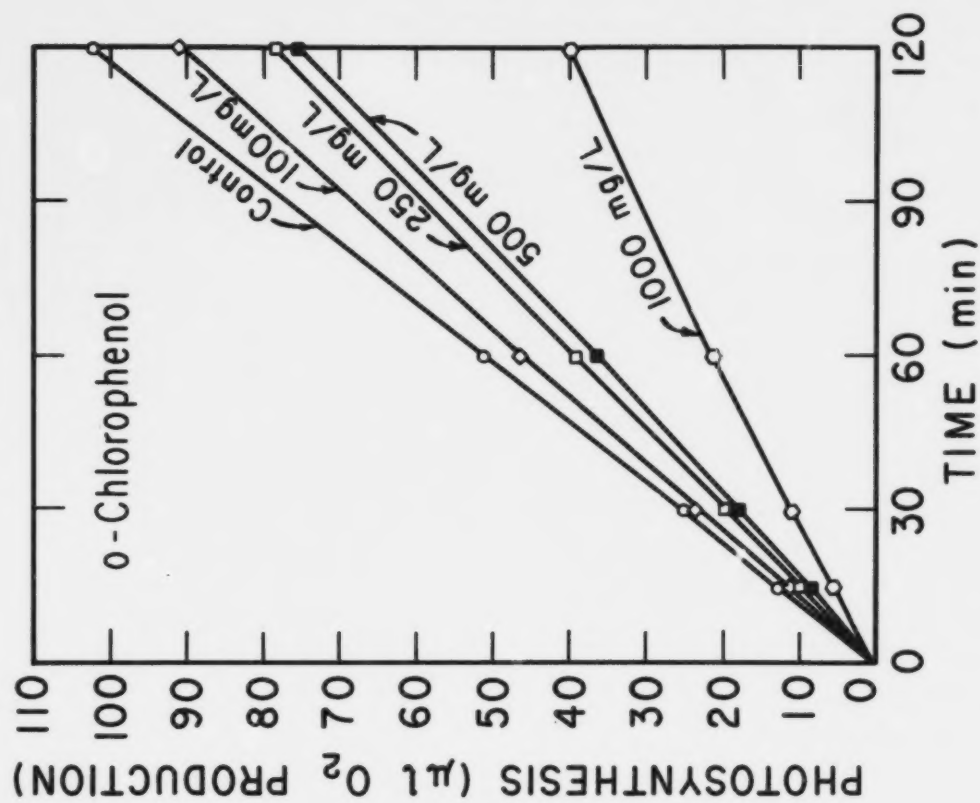


FIG. 4-2. INHIBITORY EFFECTS
OF o-CHLOROPHENOL

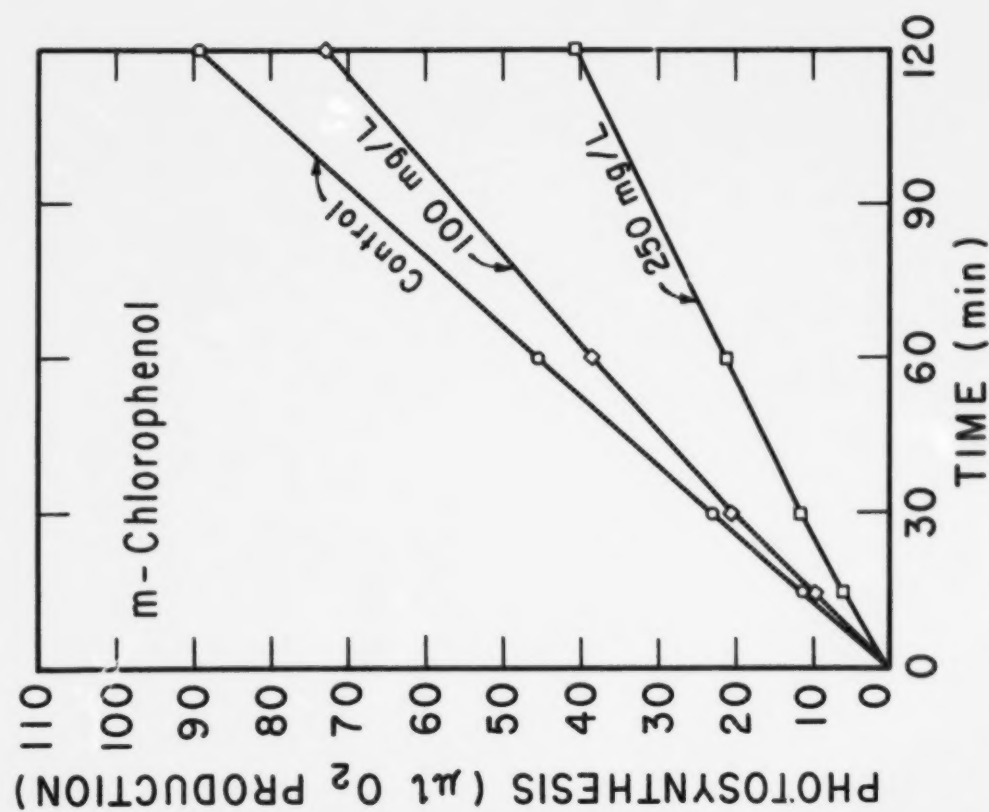


FIG. 4-3. INHIBITORY EFFECTS
OF m-CHLOROPHENOL

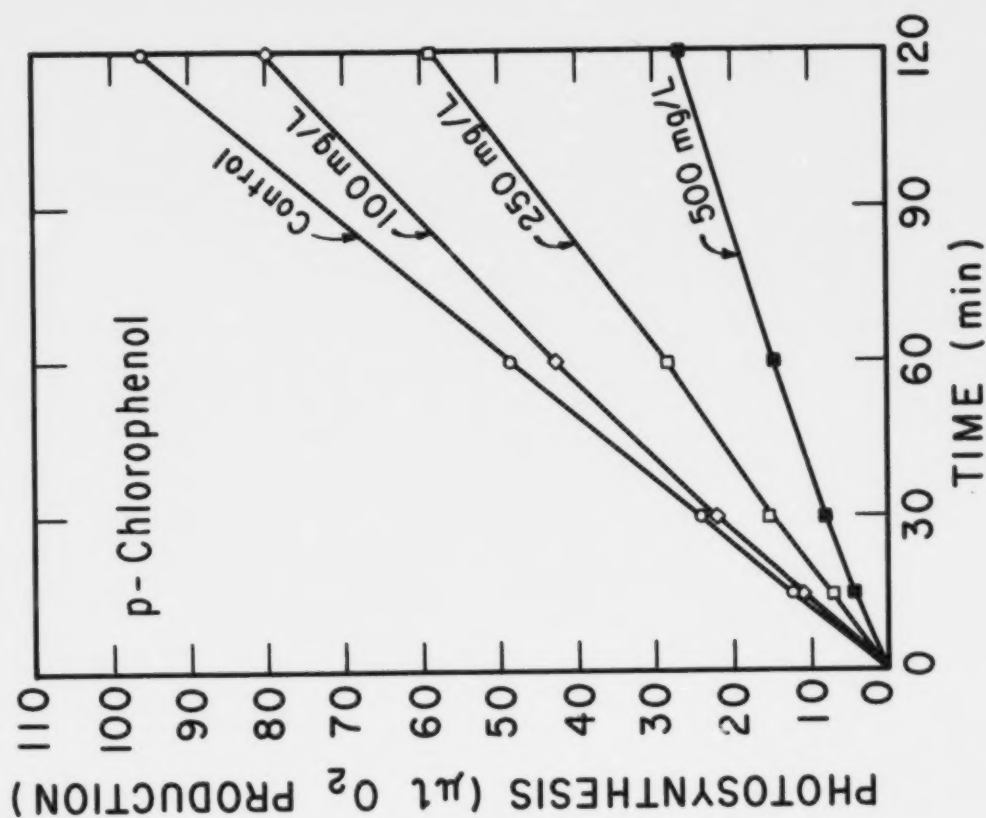


FIG. 4-4. INHIBITORY EFFECTS
OF p-CHLOROPHENOL

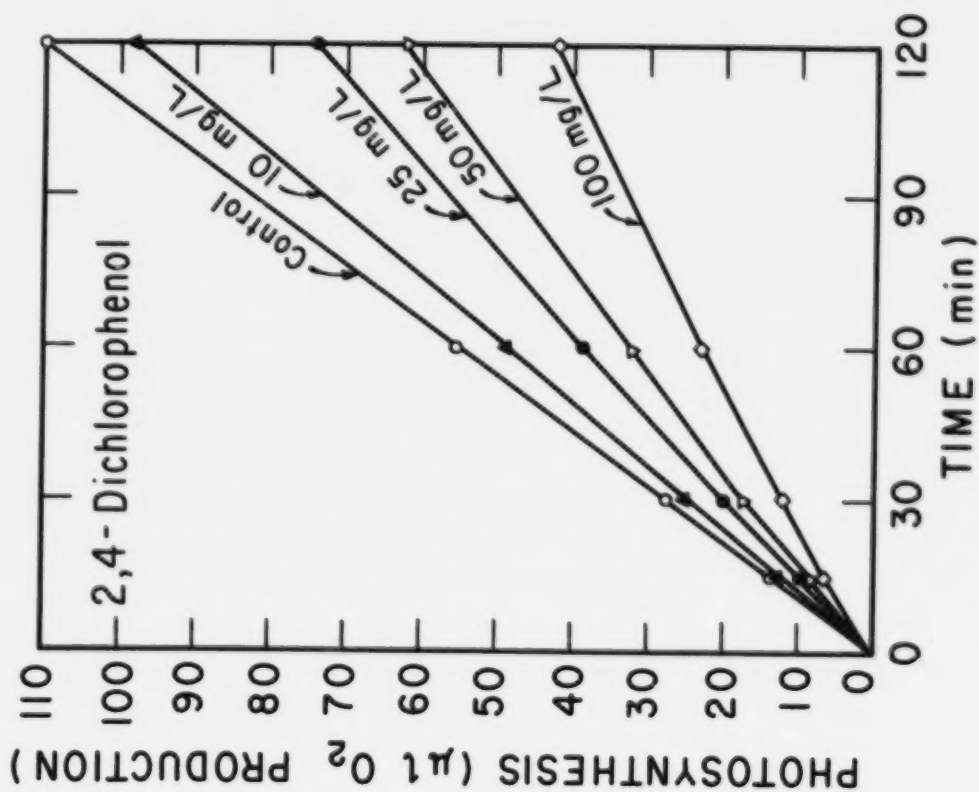


FIG. 4-5. INHIBITORY EFFECTS OF 2,4-DICHLOROPHENOL

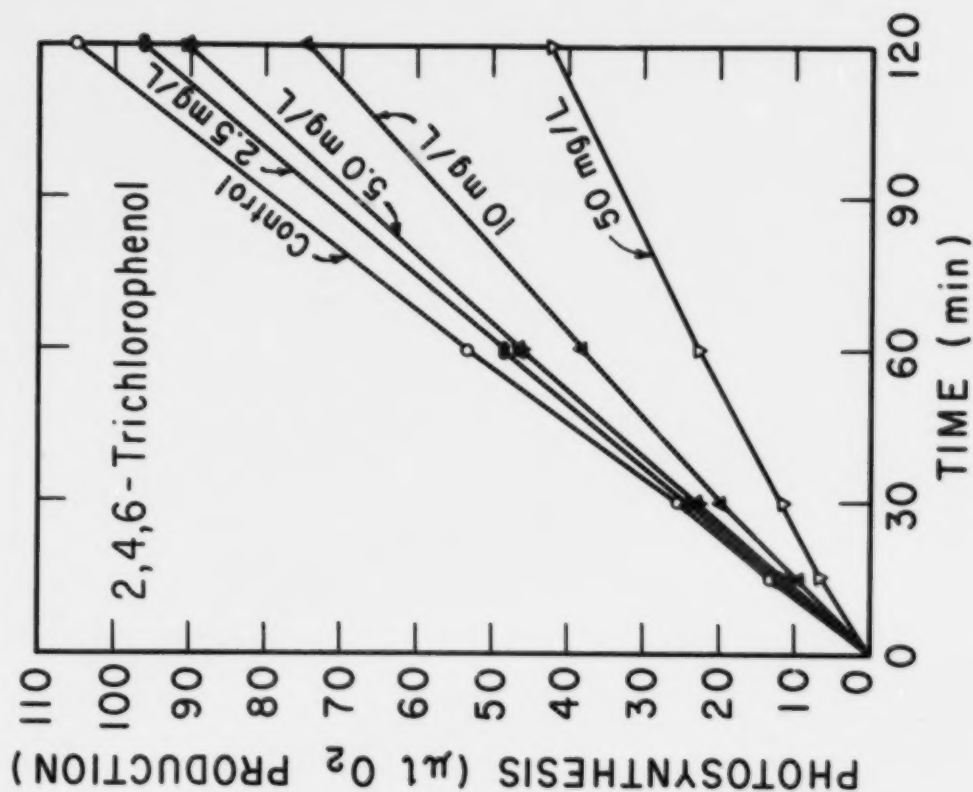


FIG. 4-6. INHIBITORY EFFECTS OF 2,4,6-TRICHLOROPHENOL

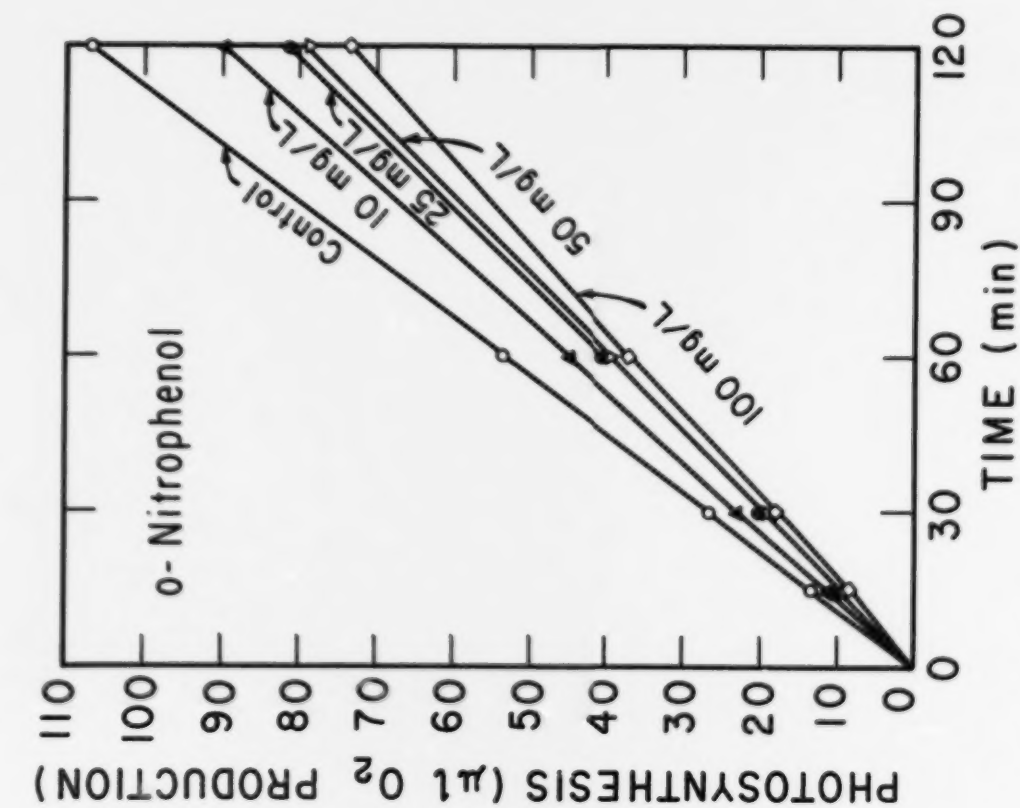


FIG. 4-7. INHIBITORY EFFECTS OF o-NITROPHENOL

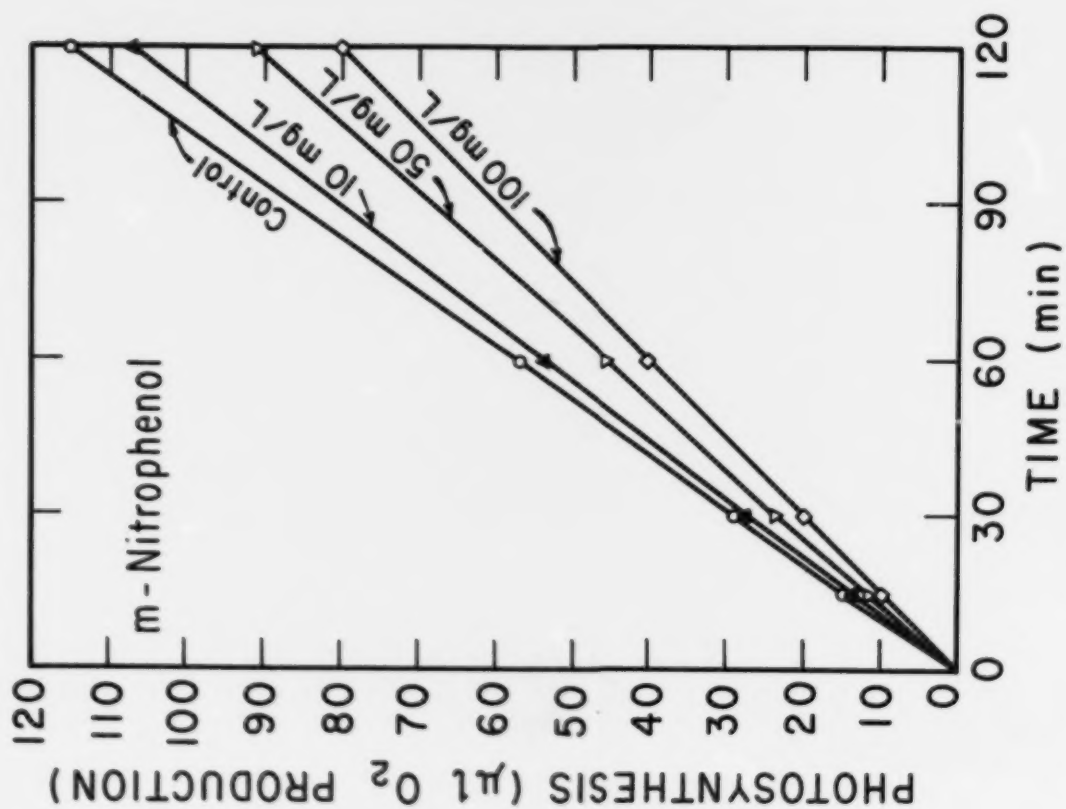


FIG. 4-8. INHIBITORY EFFECTS OF m-NITROPHENOL

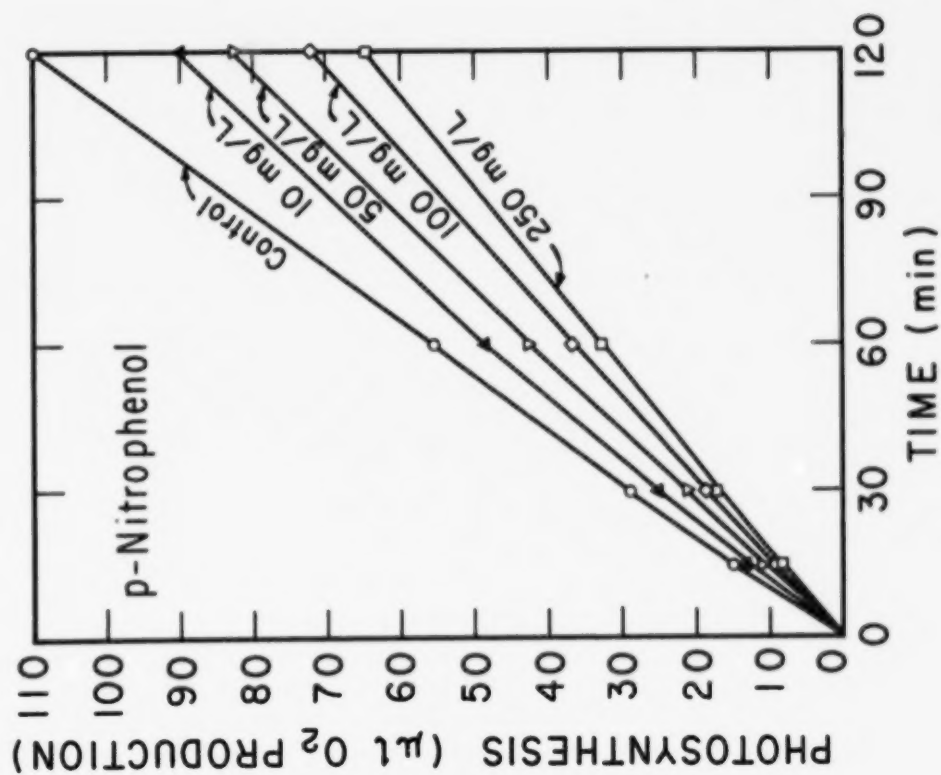


FIG. 4-9. INHIBITORY EFFECTS OF p-NITROPHENOL

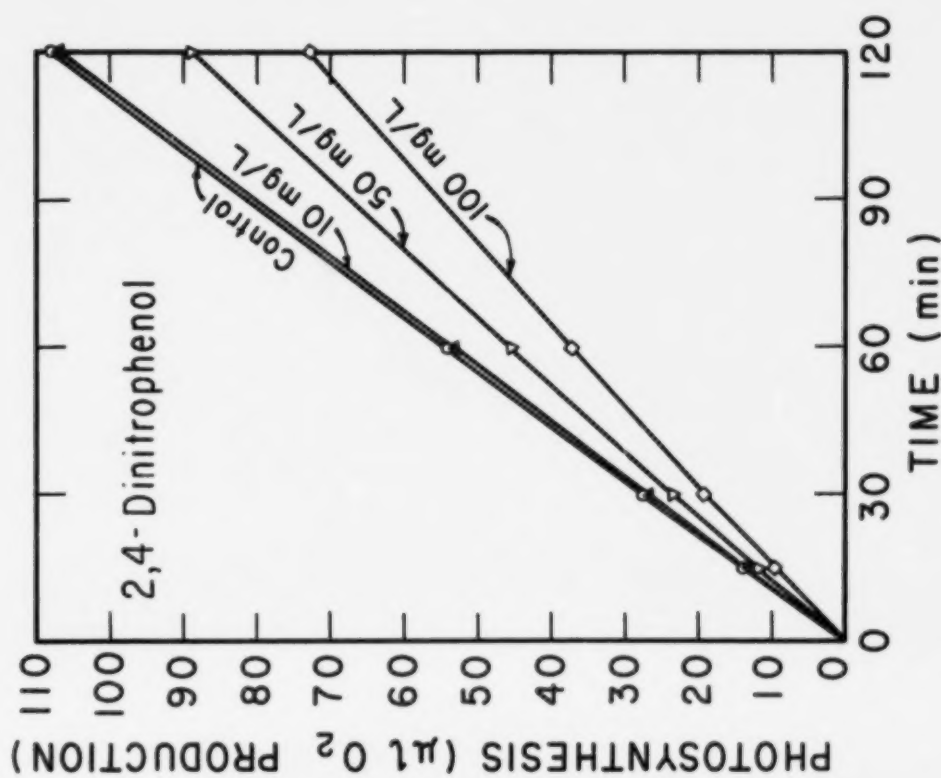


FIG. 4-10. INHIBITORY EFFECTS OF 2,4-DINITROPHENOL

Among the five chlorine-substituted phenols, the relative order of the photosynthesis-suppressing power was found to be: 2,4,6-trichlorophenol > 2,4-dichlorophenol > m-chlorophenol > p-chlorophenol > o-chlorophenol. This relative order of the photosynthesis-suppressing power was consistent with that of the effectiveness of reducing the chlorophyll synthesis during the test-tube study. For example, at the concentration of 50 mg/L 2,4,6-trichlorophenol and 2,4-dichlorophenol, respectively, reduced the rates of oxygen evolution to only 53.2 and 56.4 percent of the controls. At the concentration of 100 mg/L, the rates of oxygen production in the presence of 2,4-dichlorophenol, m-chlorophenol, p-chlorophenol and o-chlorophenol, respectively, were reduced to only 41.8, 81.7, 83.6 and 88.0 percent of the controls.

The photosynthetic activities were still maintained at 74.0 percent and 27.5 percent, respectively, of the control at 500 mg/L of o- and p-chlorophenols. However, at this level m-chlorophenol and 2,4-dichlorophenol completely inhibited the production of oxygen, as demonstrated by the negative manometer pressure readings, which also indicated that the respiratory process in algal cells was less sensitive than the photosynthetic process to the effects of toxic organics. Green *et al.* (30) also found that the amount of organic inhibitors required to inhibit respiration was larger than that required for inhibiting photosynthesis because respiration has a lower sensitivity to the inhibitors than photosynthesis.

There were no significant differences among the three isomers of nitrophenols and 2,4-dinitrophenol upon photosynthesis

during the short test period. As shown in Table 4-3, the rates of photosynthesis were reduced to 69.0, 69.5, 65.5, and 67.5 percent respectively, of the controls in the presence of 100 mg/L of o-, m-, p-nitrophenols and 2,4-dinitrophenol. At the concentration of 500 mg/L, all of these four nitro-substituted phenols completely inhibited the photosynthetic process.

CHAPTER V

MODEL WASTE STABILIZATION PONDS

It has been recognized that the presence of some toxic compounds which interfere with algal photosynthetic processes may reduce the effectiveness of waste stabilization ponds. Thirumurthi and Gloyna (73) suggested an introduction of a toxicity factor to the original Hermann-Gloya Equation for the design of waste stabilization ponds to account for the possible suppression of the photosynthetic reoxygenation.

Experimental Setup of Model Waste Stabilization Ponds

As a step toward evaluating the toxic effects of phenol on the operation of waste stabilization ponds as well as evaluating the applicability of introducing the toxicity factor into the design modification, five laboratory-controlled model ponds were assembled in the laboratory. Each pond consisted of a glass aquarium which is 60 cm long, 25 cm wide, 30 cm deep, and has a capacity of 45 liters. Each pond was baffled at three positions of equal interval to eliminate as much as possible the short-cut of the flow within the pond. The side-walls of each pond were wrapped with paper to prevent the incidences of light.

Illumination: The illumination for the ponds was provided by eight 40-watt fluorescent and four 40-watt incandescent lamps. The lamps were suspended 18 cm above the water surfaces and a masonite board coated with aluminum foil was used as reflector. A light-meter

survey indicated that incident light intensities normal to the pond surfaces ranged from 120 foot-candles to 880 foot-candles with an average value of approximately 500 foot-candles. Ponds were illuminated 12 hours a day. A timer was provided which automatically switched on the light at 6:00 AM and switched off at 6:00 PM.

Wind: Scum consisting of dead and live algal cells tended to accumulate at the water-air interface thus preventing penetration and absorption of light energy into the underlying culture. In full-scale waste stabilization ponds the formation of scum is inhibited by wind and wave action. Therefore, jets of air from 2-mm ID glass-capillary tubes were directed obliquely onto the aquaria surfaces so as to create surface currents and prevent scum formation.

Temperature: The temperature of the waste water in the model ponds was dependent upon the ambient temperature in the laboratory and absorption of radiant energy from the light sources suspended over the ponds. The average temperature of air-conditioned laboratory was 22°C. The temperature gradients for the five ponds were found to be very close. The aquaria surface temperature cycled from 22.2°C at 0600 hour when the lights were turned on, to 25.2°C at 1800 hour just before the lights were turned off. A temperature profile surveyed at the center of model ponds at various time was shown in Fig. 5-1.

Synthetic Substrate

It was anticipated that the cost of a synthetic sewage containing such items as peptone, castile soap, urea etc. was too expensive to be used on a larger scale. Therefore, a simple substrate was developed. This synthetic substrate contains non-fat dry milk

solids, or commercially called Starlac, dibasic potassium phosphate and tap water. The composition of Starlac as obtained from the manufacturer's label is shown in Table 5-1.

Table 5-1. Composition of Starlac.

<u>Food Factor</u>	<u>% Dry Weight</u>
Protein	36.0
Lactose (Milk sugar)	51.0
Milk fat	0.8
Minerals	8.2
Calcium	1.3
Phosphorus	1.0
Iron	0.0006
Sodium	0.525
Potassium	1.725
Moisture (Water)	4.0

The 5-day biological oxygen demand (BOD_5) at 20°C and the chemical oxygen demand (COD) of the Starlac powder were determined, respectively, to be 460 mg/g and 1100 mg/g. The dibasic potassium phosphate was added in proportion to the ratio K_2HPO_4 /Starlac of 93 mg/g. The dibasic potassium phosphate supplements phosphorus content, buffers the pH and also avoids the isoelectric range of such Starlac constituent as casein. Thereby, a dispersion of the liquid feed was maintained during the 24-hour period between batches. Addition of tap water insures presence of trace minerals. Table 5-2 illustrates the preparation of a synthetic substrate with a COD of 990 ng/L. The pH of this substrate is approximately 8.8. In normal practice concentrated stock substrate was prepared and kept in the refrigerator. This stock substrate could be stored for a period of four days without precipitating out casein.

Table 5-2. Preparation of a Synthetic Substrate with a COD of 990 mg/L.

<u>Ingredient</u>	<u>Quantity</u>
Starlac	0.900 gram
K ₂ HPO ₄	0.084 gram
Tap Water	added to make a total volume of one liter.

Experimental Design of Influent Waste Streams and Detention Time for Model Ponds:

The five model ponds, designated as I, II, III, IV, and V were designed to receive different concentrations of starlac substrate with different levels of phenol. The detention time for each model pond was also properly designed to meet the various purposes. The experimental design of the influent waste and the detention time for each pond were described below. The designed objectives for each pond were also included.

Pond I: This pond was used as the control. It received only starlac substrate. The designed surface organic loading of the pond was 180 pounds COD, or equivalent to 83 pounds BOD₅, per acre per day. The detention time was 15 days. Thus, this pond received 3 liters of waste with an organic strength of 990 mg/L COD. The surface organic loading and the detention time designed for this pond were quite comparable to those for field ponds used in the treatment of domestic wastes.

Pond II: In order to evaluate the toxic effects on the pond operation of 100 mg/L (dry weight) phenol present in the influent waste stream, this Pond II was designed to have the same

surface organic loading as well as the same detention time as Pond I. Therefore, Pond II also received 3 liters of influent waste with a total COD of 990 mg/L. However, in the influent waste of Pond II, 240 mg/L COD, which is equivalent to 100 mg/L dry weight of phenol, was contributed by phenol while the rest of COD was made up by starlac substrate.

Pond III: This pond was designed to receive exactly the same kind of influent waste as Pond II, but only a lesser quantity. Theoretically, it was equivalent to an expansion of Pond II for receiving the same kind and the same quantity of influent waste since the detention time in this Pond III was extended. For example, Pond III received only 2 liters of the same waste as that received by Pond II, and thus it had a detention time of 22.5 days.

As seen in the previously reported test-tube study, Chapter III, phenol at the concentration of 100 mg/L reduced the chlorophyll synthesis to about 75 percent:

$$\frac{Q_2}{Q_1} = e^{-K_{\text{phenol}} \times (C_2 - C_1)} = e^{-2.99 \times 10^{-3} \times (100-0)} = 0.74$$

where Q_1 = quantity of chlorophyll present in the control,
 Q_2 = quantity of chlorophyll present in the concentration of 100 mg/L (dry weight) phenol.

To compensate for the possible suppression of the photosynthetic capability caused by the reduction of chlorophyll synthesis, a proportionally expanded pond, or detention period was necessary. Therefore, an extended detention time was assumed:

$$\frac{15 \text{ days}}{0.74} = 20.2 \text{ days.}$$

However, since the daily influent addition was set at 2 liters, the detention was 22.5 days.

Pond IV: Ponds IV and V received 400 mg/L (dry weight) phenol in the influent waste streams. The surface organic loading of Pond IV was 290 pounds COD per acre per day. Pond IV had a detention time of 15 days and thus received 3 liters of waste daily. The influent waste contained a COD of 1620 mg/L, of which 960 mg/L COD was contributed by phenol and the rest by starlac substrate.

Pond V: This pond received exactly the same waste as Pond IV, but a lesser quantity. Similar to the explanation for Pond III, Pond V was expanded according to the fraction of the chlorophyll remaining in the tested algal culture, i.e.,

$$\frac{Q_2}{Q_1} = e^{-K_{\text{phenol}} \times (C_2 - C_1)} = e^{-2.99 \times 10^{-3} \times (400 - 0)} = 0.33$$

and hence,

$$\frac{15 \text{ days}}{0.33} = 45 \text{ days}$$

Therefore, Pond V received one liter of the same waste as compared to 3 liters supplied to Pond IV.

Influent Feedings and Effluent Withdrawals

The liquid volumes in all five ponds were maintained at 45 liters. Therefore, a certain amount of liquid was withdrawn daily. The discrepancies between the influent and the effluent quantities were due to evaporative losses.

Influent feedings and effluent withdrawals were made every morning over a period of not more than two hours.

Experimental Analyses

Approximately two and one-half months were necessary for the five model ponds to become fully acclimated and reach steady-state operation, which was characterized by uniform removals of both starlac and phenol. After the ponds had reached steady-state operation the following experimental analyses were made for a period of one month:

Total Soluble COD Remaining in the Effluents: Twice a week the effluent of each pond was centrifuged and the supernatant was analyzed for COD according to the Standard Methods (68).

Phenol Remaining in the Effluents: Coupled with COD analyses, concentrations of phenol remaining in Ponds II through V were also measured. The Direct Photometric Method (68) employing 4-amino-antipyrine was used for the measurement of phenol. The absorbance of the color formation was determined at 510 mμ by using a Beckman Spectrophotometer, Model DB, which is already described in Chapter III.

Dissolved Oxygen in the Ponds: At the end of the experiments a dissolved oxygen survey for each of the five model ponds was performed.

The levels of dissolved oxygen were monitored for each 5 cm increment of depth at the center of the ponds. The measurements were made at two-hour intervals from 0600 hour to 1800 hour.

Dissolved oxygen was measured by a Precision Scientific Company Galvanic Cell Oxygen Analyser. The galvanic cell readings were standardized with the Azide Modification of the Winkler Method (68).

Predominating Algae and Chlorophyll Contents: At the conclusion of these experiments, a microscopic examination was made to determine the predominating genus of algae in each pond. The chlorophyll content in the effluent of each pond was also analysed.

Evaluation of Analytical Results

The analytical results for each model pond are discussed under the following headings. Since the literature describes no similar experiments no comparison can be made.

Total Soluble COD Remaining in the Effluents: After the five ponds reached a relatively steady state, the soluble effluent COD and phenol concentrations remained fairly constant. The average total soluble COD and phenol remaining in the effluents of five model ponds are shown in Table 5-3. The percentage removals of both COD and phenol are also included in the table. The total soluble effluent COD was assumed to be due to the remaining phenol and starlac substrate. The COD contributed by bacterial and algal cells was neglected. The portion of COD contributed by the remaining starlac in the effluents, column h of Table 5-3, was calculated by subtracting the phenolic COD, column j, from the total soluble effluent COD, column k.

The control pond exhibited the lowest concentration of total soluble effluent COD and the highest COD removal efficiency, being 210 mg/L and 79 percent, respectively. The toxic effects of 100 mg/L (dry weight) phenol present in the influent waste stream of Pond II were demonstrated by a higher effluent COD and a lower COD removal efficiency, being 317 mg/L and 68 percent, respectively. However, the higher total soluble COD in the effluent of Pond II was notably attributed to the phenol remaining in the effluent. In Pond II the portion of the effluent COD contributed by starlac alone was 226 mg/L, which was very close to the value of 210 mg/L in the

control pond. Apparently, the residual of phenol present in Pond II did not seem to inhibit the bacterial activity.

Comparing Ponds II and III, both of which received the same kind of waste except that Pond III had a longer detention time than Pond II, the effectiveness of the compensation of the toxic effects by an expansion of the pond volume can be readily evaluated. A longer detention time of 22.5 days provided for Pond III could improve the treatment efficiency in the removals of both COD and phenol. The total soluble COD in the effluent of Pond III was 250 mg/L, which was still a bit higher than that amount of 210 mg/L of the control unit. The corresponding COD removal in Pond III was 75 percent. However, the portion of effluent COD contributed by starlac alone was only 195 mg/L, which was smaller than that in the control pond. Of course, the lower value of starlac in the effluent of Pond III than that of the control unit was due to the lower concentration of starlac added in the influent waste as well as a longer detention time provided for Pond III.

Ponds IV and V provide the same comparison as Ponds II and III. The amounts of effluent COD contributed by starlac in Ponds IV and V were respectively, 207 and 190 mg/L. Therefore, similar to what was found in Ponds II and III, the presence of 400 mg/L (dry weight) phenol in the influent waste streams did not seem to inhibit the bacterial activity. The higher values of the total soluble effluent COD for Ponds IV and V were due to the higher concentrations of phenol remaining in the effluents.

The removal efficiencies of total soluble effluent COD for Ponds IV and V were, respectively, 61 and 73 percent. Thus, the longer detention period of 45 days provided for Pond V improved appreciably the COD removal efficiency.

Phenol Remaining in the Effluents: Ponds II and III received 100 mg/L while Ponds IV and V received 400 mg/L (dry weight) phenol in the influent waste streams. As shown in Table 5-3, the concentrations of phenol remaining in the effluents of Ponds II, III, IV, and V were, respectively, 38, 23, 174 and 106 mg/L (dry weight). The corresponding removal efficiencies were 62, 73, 57, and 74 percent, respectively. Therefore, it becomes evident that a longer detention time provided for Ponds III and V could improve significantly the percentage removal of phenol.

Dissolved Oxygen in the Ponds: The dissolved oxygen profiles of the five model ponds are shown in Figs. 5-2 through 5-6. In the control pond the maximum dissolved oxygen near the water surface reached a value of approximately 12 mg/L just before the light was switched off. However, in Pond II the maximum dissolved oxygen was only 9.4 mg/L at the same time. The levels of dissolved oxygen measured in Pond II at different time were approximately 0.7 to 0.8 of that in the control unit. These data are comparable to the test-tube results. The latter indicated that phenol at a concentration of 100 mg/L (dry weight) reduced chlorophyll synthesis to 0.74 of the control. Measurement of phenol of several grab samples at different locations of Pond II indicated that the phenol concentrations ranged

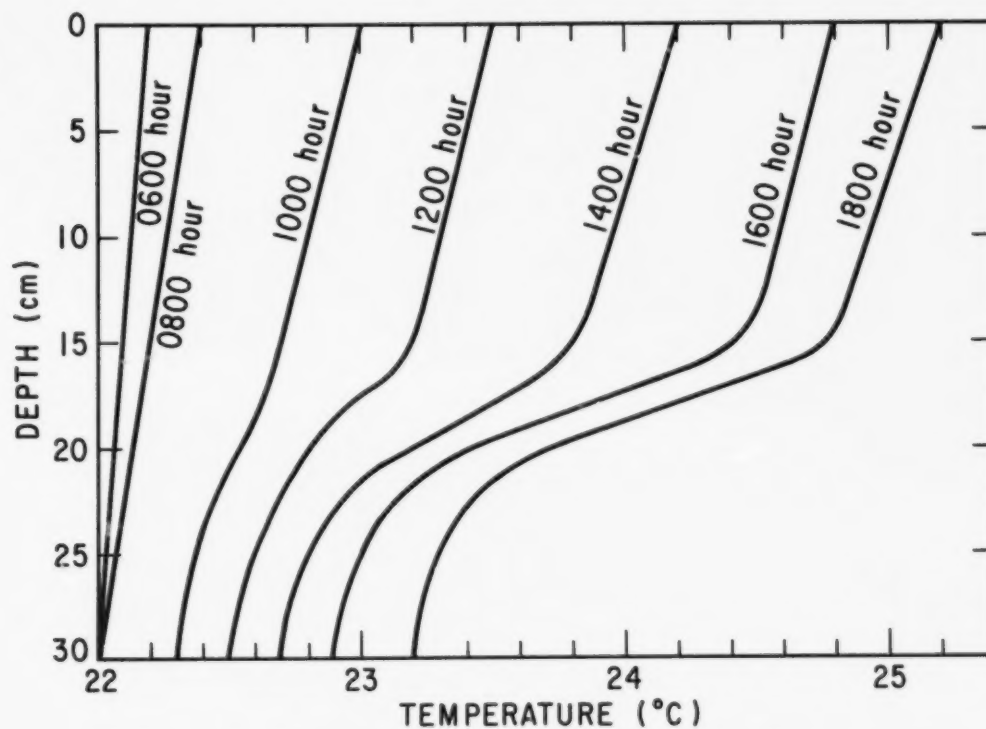
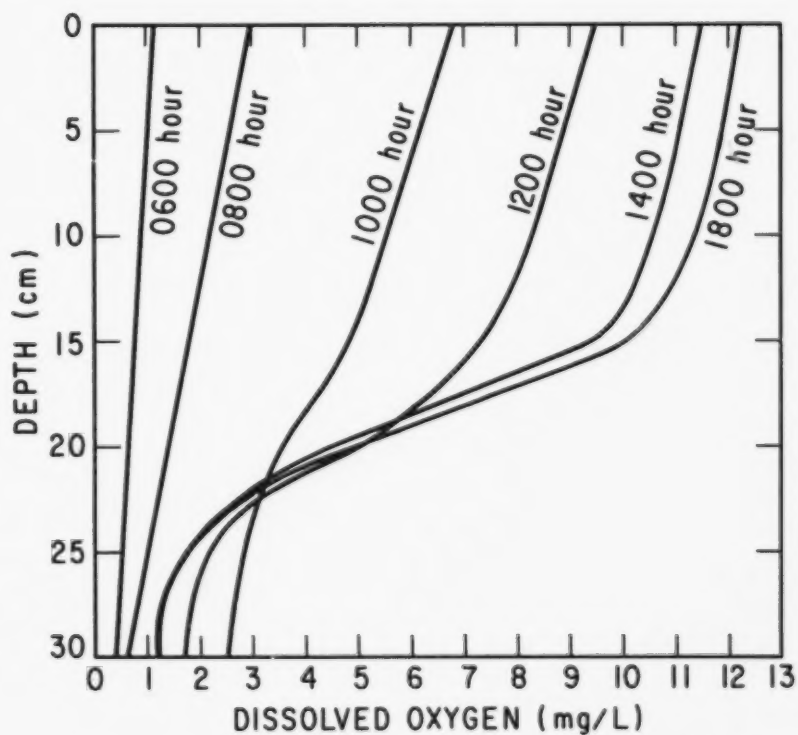


FIG. 5-1. TEMPERATURE PROFILE IN MODEL PONDS

FIG. 5-2. DISSOLVED OXYGEN PROFILE
IN MODEL POND I

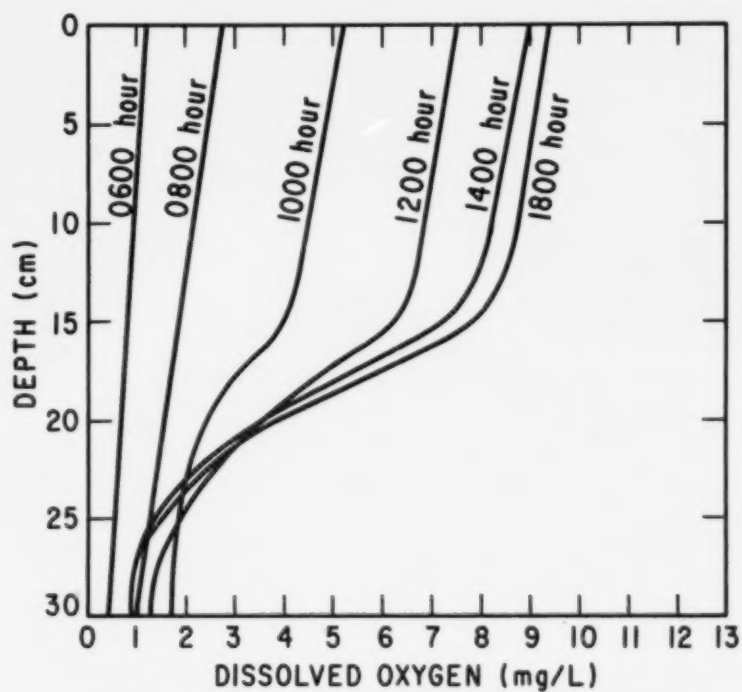


FIG. 5-3. DISSOLVED OXYGEN PROFILE
IN MODEL POND II

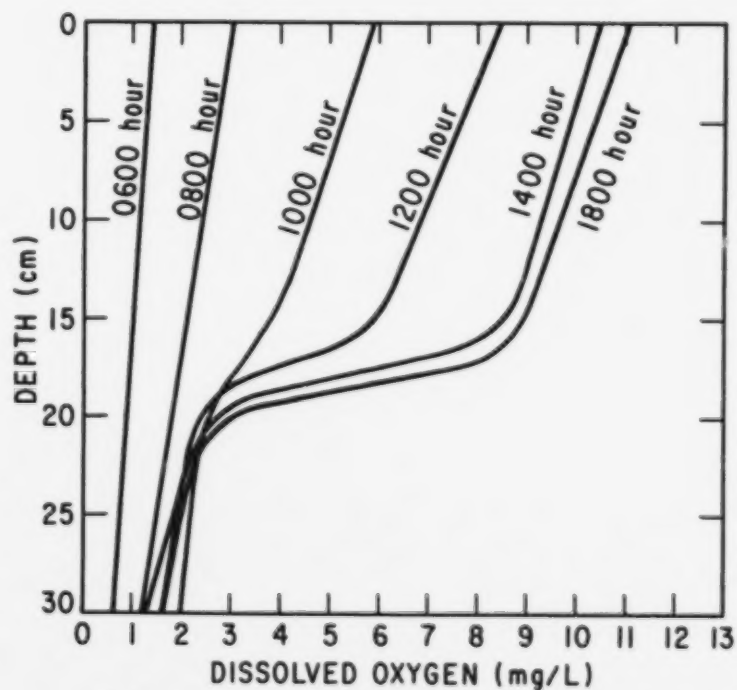


FIG. 5-4. DISSOLVED OXYGEN PROFILE
IN MODEL POND III

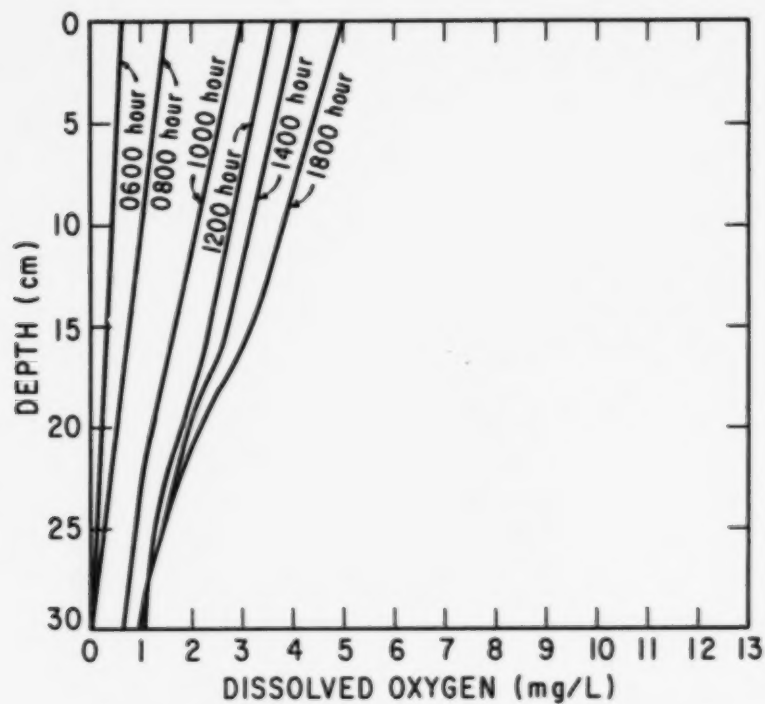


FIG. 5-5. DISSOLVED OXYGEN PROFILE
IN MODEL POND IV

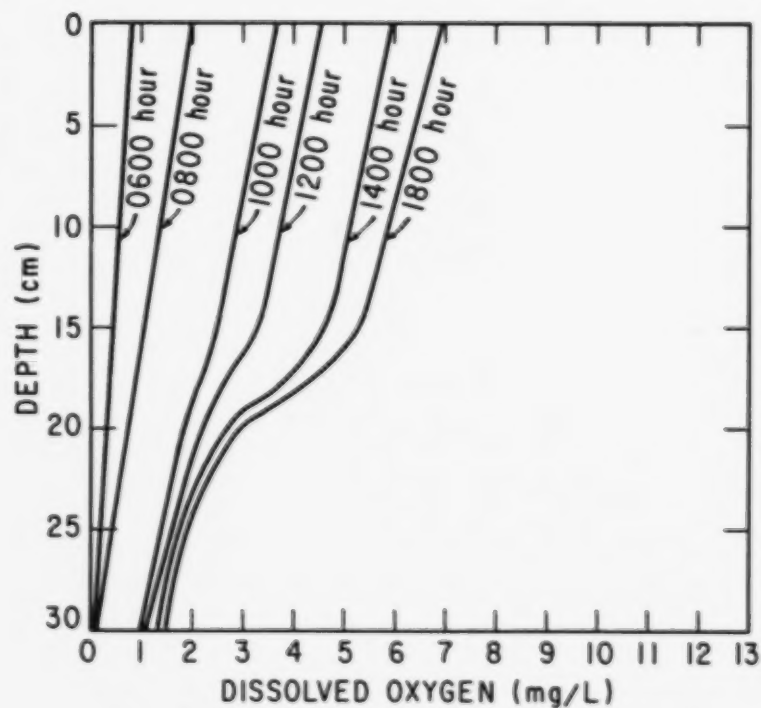


FIG. 5-6. DISSOLVED OXYGEN PROFILE
IN MODEL POND V

from 56 to 84 mg/L. Therefore, the lower levels of dissolved oxygen in Pond II were notably due to the partial inhibition of photosynthetic reoxygenation. If a higher detention time of 22.5 days was used, the dissolved oxygen levels in the pond could be raised to approximately 0.9 to 0.95 of those in the control unit, as shown in Pond III, Fig. 5-4.

In Pond IV the maximum dissolved oxygen measured was 5 mg/L, which was 41 percent of the control pond. The test-tube study indicated that 400 mg/L of phenol reduced the chlorophyll synthesis to 33 percent of the control. Measurement of phenol of several grab samples in Pond IV indicated that the concentrations of phenol ranged from 220 mg/L to 320 mg/L. Therefore, part of the phenol added to the pond was already degraded by bacteria and hence the degree of photosynthetic suppression was reduced. Although the detention in Pond V was three times as much as that in Pond IV, the dissolved oxygen in Pond V was only slightly recovered. The maximum dissolved oxygen measured was only 60 percent of that in the control unit. The low value of dissolved oxygen in Pond V was probably attributed to the higher COD in the influent and this probably overloaded the assimilative capacity of the pond system.

Predominating Algae and Chlorophyll Contents: The microscopic examination of the grab samples from each of the five ponds indicated that the predominant algal form was Chlorella in the five ponds. Only in a few occasions were Euglena detected. The exclusive predomination of Chlorella was probably attributed to the particular environmental condition of the ponds which were operating under

relatively quiescent conditions. Fogg (64) also reported that cultures containing more than one species of algae are difficult to maintain in laboratory. Although two algal species may apparently coexist in equilibrium in a natural water, almost invariably one will completely outgrow the other if brought into culture.

The chlorophyll contents in the effluents of Ponds I through V were, respectively, 2.4, 1.8, 2.0, 1.2 and 1.4 mg/L. Therefore, the percentages of the chlorophyll contents in Ponds II through V, as compared to the control pond, were respectively, 0.75, 0.84, 0.50, and 0.59. These figures are quite comparable with those of the dissolved oxygen levels which are described in the previous section.

CHAPTER VI

DISCUSSION

The low cost waste treatment device of the waste stabilization pond has attracted more popularity in the treatment of industrial wastes. However, the assimilative capacity of waste stabilization ponds is affected when the photosynthetic capabilities of algae are reduced. This chapter discusses the relationship between the toxicity and chemical structure of phenolic-type compounds, possible design modifications of waste stabilization ponds and limitations of the toxicity data obtained in this investigation when applied in design modifications.

Relationship between the Toxicity and Chemical Structure of Phenolic-Type Compounds

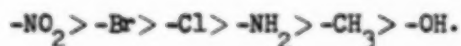
For the majority of phenolic-type organics it was found that the chlorophyll reductions were generally logarithmic with respect to the concentration of the compound present. Therefore, the slope of the line which relates the chlorophyll reduction with the increasing concentration is defined as the toxicity constant, K . Furthermore, the relative magnitude of any toxicity can be defined in terms of the phenol coefficient, $\frac{\text{toxicity of a compound}}{\text{toxicity of phenol}}$ or $\frac{K_{\text{compound}}}{K_{\text{phenol}}}$. The toxicity constants and phenol coefficients for the tested phenolic-type compounds are summarized in Table 6-1.

From Table 6-1, it is evident that the toxicity of phenolic-type compounds is a function of the substituent group as well as the

Table 6-1. Toxicity Constants of Selected Compounds

Organic Compounds	Toxicity Constant ($\times 10^{-3}$)	Phenol Coefficient	Concentration Tested Showing No Toxicity (mg/L)
Phenol	2.99	1.00	-
o-Bromophenol	8.88	2.97	10.0
m-Bromophenol	20.53	6.85	10.0
p-Bromophenol	18.75	6.28	10.0
o-Chlorophenol	7.18	2.40	10.0
m-Chlorophenol	18.12	6.06	10.0
p-Chlorophenol	15.28	5.11	10.0
2,4-Dichlorophenol	32.70	10.90	1.0
2,4,5-Trichlorophenol	472.00	158.00	1.0
2,4,6-Trichlorophenol	412.00	138.00	1.0
Pentachlorophenol	656000.00	222000.00	-
o-Cresol	4.41	1.48	-
m-Cresol	4.06	1.36	-
p-Cresol	4.69	1.57	-
2,4-Xylenol	14.08	-	50.0
2,5-Xylenol	12.20	-	50.0
2,6-Xylenol	8.60	-	50.0
3,4-Xylenol	10.96	-	50.0
3,5-Xylenol	9.86	-	50.0
o-Nitrophenol	62.00	20.75	1.0
m-Nitrophenol	49.60	16.61	1.0
p-Nitrophenol	77.40	25.90	1.0
2,4-Dinitrophenol	66.30	-	10.0
4,6-Dinitro-o-cresol	41.10	-	10.0
o-Aminophenol	14.65	4.90	-
m-Aminophenol	4.92	1.65	-
p-Aminophenol	5.70	1.91	-
2-Amino-1-phenol-4-sulfonic acid	2.06	-	1000.0
Catechol	2.22	0.74	-
Resorcinol	1.21	0.41	-
Hydroquinone	3.89	1.30	-
Pyrogallol	-	-	-
Phloroglucinol	-	-	-
DDT	-	-	saturation
Lindane	296.00	99.00	-
2,4-D	4.74	1.58	5.0
Sodium salt of 2,4-D	4.98	1.67	-
2,4,5-T	5.65	1.89	20.0
Sodium TCA	0.05	0.017	500.0
Dow Esteron 99	0.39	0.13	-
Dow Formula 40	0.28	0.094	-

relative substitution position. Introduction to the phenol nucleus of any substituent group other than a hydroxyl group increases the toxicity. The effectiveness of the substituent group in raising the toxicity decreases in the following order:

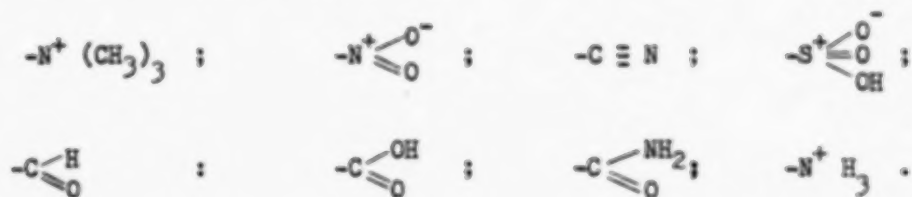


The additional introduction of another substituted group generally further increases the toxicity. For example, among the chlorine-substituted phenols the degree of toxicity decreases in the order of: pentachlorophenol > trichlorophenols > dichlorophenol > monochlorophenols. This is the same case for methyl-substituted phenols since xylenols have higher toxicities than cresols at concentrations above 100 mg/L. However, nitro-substituted and hydroxyl-substituted phenols do not follow this rule. Para and ortho-substituted phenols generally have a higher toxicity than the meta-isomer. However, halogen-substituted phenols are more toxic in meta-substitutions.

The above relationship between the chemical structure and the toxicity may probably be explained on the basis of structure. There are generally two types of reactions involved in the aromatic substitutions: abnormal and normal reactions. The abnormal type of reaction is reversible and markedly subject to effects of temperature as well as any steric factor. Some of abnormal types of reactions include sulfonation, alkylation and acylation. The normal type of reaction is an irreversible process and not subject to the influences of temperature. Nitration and halogenation of the aromatic nucleus belong to this normal type of reaction.

Substituted compounds formed by the normal type of reaction are expected to be more stable and thus have higher toxicity. This probably explains the fact that nitrophenols and halogenophenols are more toxic to Chlorella pyrenoidosa than other phenolic-type compounds investigated in this study.

The rules of orientation and ease of introduction of an entering substituent, which are controlled by the substituent group already present in the benzene ring, can also be applied to predict the stability of the compound and thus explain why ortho- and para-substituted phenols are more toxic than the meta-isomers. In general, the substituent group can be classified as two types: meta directing group and ortho-para directing group. The meta directing group is the one in which the atom directly joined to the aromatic nucleus is either strongly unsaturated or positively charged. This meta directing group will deactivate all the positions, with particular suppression of attack at the ortho and para carbon atoms, and permit only with difficulty the substitution on the meta position. Several of the meta directing groups are:

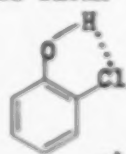


The ortho-para directing group is the one in which the atom directly joined to the aromatic nucleus is saturated. This group will facilitate further substitution and direct the entering substituents to preferably para or ortho position. The chief groups of this type are:

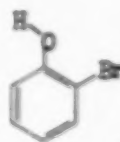
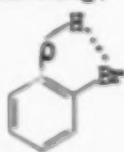


The above rules of orientation seem to be useful for predicting the ease of substitution reactions as well as the stability of the resulted compound.

Phenol has a very powerful ortho-para directing hydroxyl group, therefore any further introduction of a ortho-para directing group such as $-\text{CH}_3$ and $-\text{NH}_2$ to phenol will be directed preferably to the para or ortho position to form a favorable and stable configuration. Therefore, para- and ortho-isomers of cresols and aminophenols exhibit higher toxicities than the meta-isomers. However, halogenated phenols do not follow these rules since the meta- and para-isomers of halogenophenols are more toxic than the ortho-isomers. This is notably due to the possible formation of hydrogen bondings in the ortho-isomers and hence suppresses the activity of the halogen atoms. For example, o-bromophenol can exist in both cis- and trans-forms of isomers while o-chlorophenol can exist entirely in the cis-form. The formation of hydrogen bondings are absolutely restricted only to the cis-forms isomers, as shown in the following:



cis-forms



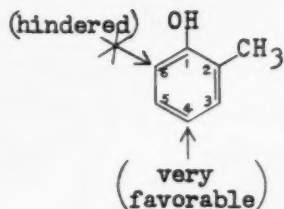
trans-form

The above property of the hydrogen bondings formation also partly accounts for the fact that o-bromophenol has a higher toxicity than o-chlorophenol.

In case of the introduction of a meta directing group to phenol, the orientation of the entering group is also determined by the directing influences of the hydroxyl group already present in phenol. Therefore, upon the introduction of a nitro group to phenol, the nitro substituent is favorably directed to the ortho or para position. As a matter of fact, in the nitration of phenol, p- and o-nitrophenols are formed, respectively, about 60 and 40 percent. Thus, para- and ortho-nitrophenols are probably more stable than the meta-isomer and hence exhibit relatively higher toxicities.

For the poly-substituted phenols such as xylenols, the same rules as described in the above paragraphs hold. Since -OH has higher

directing influence power than $-CH_3$, methylation of o-cresol is favorably directed to the position 4, which is para to -OH and meta to $-CH_3$. This explains why 2,4-xyleneol is more toxic than any other xylenols tested. The addition of a methyl group to the position 6, even which is ortho to -OH and meta to $-CH_3$, however, is very unfavorable and highly reversible because the position 6 is subject to the steric hindrance (17).



3,4-xyleneol is more toxic than 3,5-xyleneol because one of the methyl substituents in 3,4-xyleneol is para to -OH group while both of the methyl substituents in 3,5-xyleneol are meta to -OH.

Design Modifications of Waste Stabilization Ponds

The assimilative capacity of waste stabilization ponds is subject to the influences of the photosynthetic reoxygenation of algae. Toxic compounds present in waste streams may affect the

operation of waste stabilization ponds in at least four ways.

These are listed below.

a.) Toxic compounds may inhibit the photosynthetic process and hence suppress algal reoxygenation in the pond systems. Consequently, aerobic conditions may be difficult to maintain.

b.) Toxic compounds may inhibit the bacterial activity. The rate of bio-degradation of organic compounds is reduced. Therefore, ponds become less efficient in the treatment of wastes.

c.) The biodynamic equilibrium of biological populations in the pond may be upset by the presence of toxic compounds.

d.) Most of toxic compounds are relatively resistant to bio-degradation. Unoxidized toxic compounds frequently impose appreciable fraction of COD remaining in the effluents. Consequently, the overall treatment efficiency of the pond is reduced.

Most design equations for the waste stabilization ponds are based, either theoretically or empirically, on the treatment of domestic-like wastes which have high biodegradation coefficients. The ponds receiving industrial wastes are likely to be under-designed if common reaction ratios are used.

The model studies involving waste stabilization ponds substantiate the predicted decrease in dissolved oxygen as well as the higher value of COD remaining in the effluents owing to the contribution of the unoxidized toxic compound.

It is reasonable to assume that the photosynthetic capability is directly related to the amount of chlorophyll present in an algal suspension. Toxic compounds inhibiting the chlorophyll

synthesis will eventually suppress the photosynthetic oxygenation. Consequently, the toxic data related to the inhibition of chlorophyll formation as found in this research can probably be used to improve the existing design equations, particularly when certain industrially produced organic wastes are present.

Hermann and Gloyna (32) and as modified by Gloyna (28) suggested a relationship for the design of domestic ponds. Thirumurthi and Gloyna (73) suggested that this concept may also be used as a basis for industrial pond design provided that a toxicity factor is introduced into the design equations to properly expand the pond volume and bring up the assimilative capacity inhibited by the toxic compounds. Whereas the earlier designs were based on a depth of 3.5 feet, it is now recognized that a depth of 6.0 feet is more desirable in temperate and colder climates. Consequently, for domestic-like waste streams an acceptable design equation might be as follows:

- a.) Using the original Hermann-Gloyna equation for a depth of 3.5 feet,

$$V = 5.37 \times 10^{-8} \times N \cdot q \cdot Y \times 1.072^{(35-T)} \quad \text{.....Eq. 6-1}$$

where V = designed volume of the pond in acre-feet,
 N = number of people served,
 q = daily per capita sewage volume in gallons/day,
 $(N \cdot q)$ = volume of daily influent waste flow in gallons/day),
 Y = influent BOD in mg/L,
 T = temperature in °C (based on the minimum monthly temperature).

- b.) Using the modified version for a depth of 6.0 feet,

$$V = 9.20 \times 10^{-8} \times N \cdot q \cdot Y \times 1.072^{(35-T)} \quad \text{.....Eq. 6-2}$$

Now if waste streams contain industrially produced organics which have inhibitory effects on the chlorophyll formation and hence photosynthetic oxygenation, the designed volume as shown in Eqs. 6-1 and 6-2 should be properly increased to maintain the desired treatment efficiency of the pond. The expansion of the pond volume may perhaps be made according to the extent of the inhibition of the chlorophyll synthesis.

For most of the phenolic-type compounds and certain pesticides selected in this study, the reduction of chlorophyll content is generally logarithmic to the concentration of the toxic compound. This relationship may also probably be true for the other toxic compounds not included in this investigation. Therefore, when an algal suspension is exposed to a toxic compound, the fraction of the chlorophyll remaining, as compared to the same algal suspension without having the toxic compound, can be expressed in the following relationship:

$$\text{Fraction of chlorophyll remaining} = \frac{Q}{Q_0} = e^{-K \cdot C} \dots\dots\dots \text{Eq. 6-3}$$

where Q_0 = chlorophyll content if there is no toxic compound present,
 Q = chlorophyll content in the presence of the toxic compound having a concentration of C ,
 K = toxicity constant of the toxic compound,
 C = concentration of the toxic compound.

If the toxicity constant is known, the fraction of chlorophyll remaining can thus be calculated. Table 6-1 lists the toxicity constants for the compounds selected in this investigation.

If there are more than one toxic chemical present in the influent waste stream, the fraction of chlorophyll remaining becomes:

$$\text{Fraction of chlorophyll remaining} = e^{-\sum_{i=1}^I K_1 \cdot C_1} \dots\dots\dots \text{Eq. 6-4}$$

where K_1 and C_1 are, respectively, the toxicity constant and the concentration of the i^{th} toxic compound in the waste stream.

Or more practically, the original waste sample is employed directly for the determination of the toxicity constant of the waste itself, and then the fraction of chlorophyll remaining is predicted by using Eq. 6-3.

Based on the above argument, if ponds receive waste streams which contain industrially produced organics, Eq. 6-2 can be rewritten to produce Eq. 6-5.

$$V = 9.20 \times 10^{-8} \times N \cdot q \cdot Y \times 1.072^{(35-T)} \times e^{\sum_{i=1}^I K_1 \cdot C_1} \dots\dots\dots \text{Eq. 6-5}$$

It must be borne in mind that in the above modification, the inhibitory effects of bacterial activity and the variations in biodegradability of the waste are not taken into consideration. Also, part of the toxic organic chemical is subject to bio-degradation. Under a complete mixing and continuous flow condition, the average concentration of the organic (or toxic) compound remaining in a pond can be expressed in Eq. 6-6 (48).

$$C = \frac{C_0}{(V/Q)} \int_{t'=0}^{\infty} e^{-kt'} e^{-(Q/V)t'} dt' = \frac{C_0}{k(V/Q) + 1} \dots\dots\dots \text{Eq. 6-6}$$

where C = average concentration of the organic (or toxic) compound remaining in the pond, or equal to the concentration remaining in the effluent,
 C_0 = original concentration of the organic (or toxic) compound present in the influent waste stream,
 V = volume of the pond,
 Q = quantity of influent flow,
 e = base of the natural logarithmic system,
 t' = time of exposure (i.e., detention),
 k = rate constant of bio-degradation of the organic (or toxic) compound.

Since V/Q = reaction time, t , of the pond, Eq. 6-6 can be rewritten into Eq. 6-7.

$$C = \frac{C_0}{kt + 1} \dots\dots\dots \text{Eq. 6-7}$$

Thus, the reaction time required for a given treatment can be calculated from Eq. 6-7. As shown in this equation, the reaction time required depends upon the rate constant of bio-degradation of the waste and the degree of treatment intended. The rate constant is in turn dependent upon the temperature and the biodegradability of the waste. Equation 6-8 relates the reaction time required for a given treatment with rate constant and temperature.

$$\frac{t}{t_0} = \frac{k_0}{k} = \theta^{(T_0 - T)} \dots\dots\dots \text{Eq. 6-8}$$

where t_0 and t = reaction times required (for a given treatment) at rate constants of k_0 and k , respectively (i.e., bacteriological action),
 k_0 and k = rate constants (bacteriological degradation) at temperatures of T_0 and T , respectively,
 θ = temperature coefficient for a specific waste.

Noteworthy is the fact that values of k_0 , k , and θ are dependent upon the biodegradability of the specific waste.

Equation 6-8 can be rewritten as Eq. 6-9.

$$t = \frac{k_0}{k} t_0 \dots\dots\dots \text{Eq. 6-9}$$

Substituting Eq. 6-9 into Eq. 6-7, it becomes

$$C = \frac{C_0}{k_0 t_0 + 1} \dots\dots\dots \text{Eq. 6-10}$$

Therefore, a toxicity-compensation factor (for the suppression of photosynthetic reoxygenation) can be shown in the following form:

$$\text{Toxicity-compensation factor} = f = \frac{K_1 \cdot C_1}{\theta^{(T_0 - T)} k_0 t_0 + 1} \dots\dots\dots \text{Eq. 6-11}$$

(for photosynthetic reoxygenation)

where K_1 = toxicity constant (algal oxygenation) of the i^{th} toxic compound,
 k_{o1} = observed rate constant of biodegradation of the i^{th} toxic compound at T_o .

Based on quantity of waste flow, reaction time, rate constants of biodegradation of the waste and the toxic compound, biochemical oxygen demand, and algal toxicity-compensation factor, a general design equation with a rational basis can be developed for determining the volume of an industrial waste stabilization pond:

$$V = Q \cdot \frac{Y}{200} \cdot \left(\frac{k_o}{k} \right) t_o \cdot e^{\sum_{i=1}^i \frac{K_1 C_{o1}}{k_{o1} t_o + 1}} \dots \dots \dots \text{Eq. 6-12}$$

where V = designed volume of the pond,
 Q = quantity of influent flow per day,
 Y = 5-day 20°C BOD,
 k_o = observed rate constant of biodegradation of the waste at T_o ,
 k = expected rate constant of biodegradation of the waste at T ,
 (T is based on the minimum monthly temperature),
 t_o = reaction time required for a given treatment at a rate constant k_o , (t_o can be calculated according to Eq. 6-7),
 K_1 = toxicity constant (algal oxygenation) of the i^{th} toxic compound,
 C_{o1} = original concentration of the i^{th} toxic compound present in the influent waste stream,
 k_{o1} = observed rate constant of bio-degradation of the i^{th} toxic compound at T_o .

The figure of 200 present in Eq. 6-12 is the average 5-day BOD strength of domestic sewage in the United States (32). Certain limitations seem to occur in the application of Eq. 6-12 since the evaluation of the biodegradation of the individual toxic compound in the waste stream seems to be impractical. Therefore, it is recommended that the evaluation of the toxicity constant (algal oxygenation) is based on the waste itself. Thus, Eq. 6-12 becomes:

$$V = Q \cdot \frac{Y}{200} \cdot \left(\frac{k_o}{k} \right) t_o \cdot e^{K \cdot \left(\frac{C_o}{k_o t_o + 1} \right)} \dots \text{Eq. 6-13}$$

where K = toxicity constant (algal oxygenation) of the waste itself,
 k_o = observed rate constant of biodegradation of the waste at T_o ,
 C_o = original concentration of the influent waste.

In applying Eq. 6-13, it is suggested that the original waste is first diluted to a factor of $(k_o t_o + 1)$. Then, the toxicity of this diluted waste, which now has a concentration of only $\frac{C_o}{k_o t_o + 1}$, to algae is determined.

Furthermore, by substituting Eq. 6-8 into Eq. 6-13, it is possible to rewrite the design equation in the following form:

$$V = Q \cdot \frac{Y}{200} \cdot [\theta^{(T_o - T)}] \cdot t_o \cdot e^{K \cdot \left(\frac{C_o}{k_o t_o + 1} \right)} \dots \text{Eq. 6-14}$$

If the practical units are used, Eq. 6-14 can be rewritten to produce Eq. 6-15.

$$V = 1.54 \times 10^{-8} \cdot Q \cdot Y \cdot [\theta^{(T_o - T)}] \cdot t_o \cdot e^{K \cdot \left(\frac{C_o}{k_o t_o + 1} \right)} \dots \text{Eq. 6-15}$$

where V = designed volume of the pond in acre-feet,
 Q = quantity of influent flow in gallons/day,
 Y = 5-day 20°C BOD in mg/L,
 θ = temperature coefficient, depending upon the specific waste, θ being 1.085 for certain industrial wastes at 35°C,
 T_o = observed maximum operation temperature (based on the maximum monthly temperature), °C,
 T = expected minimum operation temperature (based on the minimum monthly temperature), °C,
 t_o = reaction time required for a given treatment at T_o and k_o ,
 e = base of the natural logarithmic system,
 K = toxicity constant (algal oxygenation) of the waste itself,
 C_o = original concentration of the waste, mg/L dry weight, or mg/L BOD,
 k_o = observed rate constant of biodegradation of the waste at T_o .

Limitations of K Values

The toxicity of a chemical to typical microorganisms in aquatic environments depends on various factors such as the type and growth habits of the organisms, the concentration and molecular structure of the chemical and the environmental conditions. Neither these factors nor their interrelationships are well defined. Hence, it is practically impossible to simulate field conditions in the laboratory. In a laboratory test program it is necessary to limit the interacting variables as much as possible.

Toxicity data of selected compounds reported in this dissertation are obtained from the experiment conducted with pure cultures of Chlorella pyrenoidosa under defined environmental conditions. Hence, the toxicity values are relative. These data yield specific information which is important in the understanding and managing of ecological systems. Although actual model ponds studies have reflected the applicability of the design modification, the laboratory-controlled model ponds were different from those field stabilization ponds in several respects such as light intensity and quality, temperature variations, wave actions, diversities of bio-populations, predominant algal form, and pond sediment etc. Therefore, it is suggested that field pilot-plant studies should be conducted before laboratory-determined toxicity data can be extrapolated for use in natural environments. It can be expected that under certain ecological conditions particularly favorable to the growth of algae, or where the pollutants are increased gradually, algae may probably survive at a higher toxic concentration than that found in the laboratory (74). For example, it was reported that copper ore,

at concentrations that appeared promising in laboratory experiments was not useful in the treatment of natural waters to suppress harmful flagellate blooms.

Also, it should be noted that algae possess considerable powers of adaptation to toxic compounds. Fogg (23) reported that by subculturing the blue-green alga Anacystis nidulans in gradually increasing concentrations of antimetabolites and antibiotics it has been able to produce strains resistant to their toxic effects. Such adapted strains did not show greatly reduced relative growth rate.

It must be emphasized that the effects of environmental conditions on the stability and potency of toxicants play an important role in the interpretation of results. Fitzgerald (22) found that 2,3-dichloronaphthoquinone is toxic to blue-green algae at a concentration as low as 2 $\mu\text{g/L}$. However, under alkaline conditions this chemical is hydrolyzed to 2-chloro-3-hydroxy-naphthoquinone, which is not an effective algicide at concentrations of 1 to 2 mg/L . It was also reported (74) that lindane decays in sea water (70 percent after 30 days) and that a freshly prepared pesticide solution of Nabam is more toxic than an aged solution.

APPENDICES

APPENDIX I

CHLOROPHYLL EXTRACTION

The detailed procedure of chlorophyll extraction by using hot methanol is described below:

Step 1.

Transfer with a pipette 2.5 ml of test algal suspension into a centrifuge tube. Centrifuge for 15 minutes at 3,400 rpm, 24 cm diameter head. This provides a centrifugal force of approximately 1,550 g.

After centrifuging, decant the supernatant thoroughly; add approximately 3 ml of distilled water, suspend and wash algal residue; centrifuge again for 15 minutes at 3,400 rpm. After the second centrifuging, decant the supernatant, leaving approximately 0.25 ml of water and algal cells.

Step 2.

Suspend the cells by using a Precision Scientific Company Super Mixer. If the Super Mixer is not available, suspend the cells by rapidly thumping centrifuge tube with forefinger. Do not stir with stirring rod; be careful to maintain a firm grip on lip of centrifuge tube with thumb and forefinger of the other hand to prevent the slip of the tube while thumping.

Coagulate suspended algal cells by heating in a boiling water bath for 30 to 45 seconds depending on the quantity of algal cells and water left in the centrifuge tube.

Step 3.

Add 2.0 to 2.5 ml of methanol and stir, removing hardened algae from side of centrifuge tube. Heat in boiling water bath for 15 seconds. The tube should be partially submerged, withdrawn and shaken constantly. Permit the contents to cool for at least 5 minutes before proceeding further.

Heat in boiling water bath with constant shaking. Do not permit the contents to boil over. Continue until approximately 0.5 ml of the extract remains. Centrifuge for 5 minutes at 3,400 rpm and filter extract through medium sintered glass filter. Wash lip of centrifuge tube and filter with not more than 3 ml of 80 percent by volume acetone-water solution.

Step 4.

Perform the second extraction of chlorophyll by repeating Step 3. For complete removal of chlorophyll, wash side of centrifuge tube and suspend cells with one ml of 80 percent acetone-water solution. Centrifuge for 5 minutes at 3,400 rpm and filter the supernatant. Wash lip of centrifuge tube and filter with not more than 1.5 ml of 80 percent acetone-water solution.

Transfer filtered extract to 10 ml graduate cylinder, wash centrifuge tube with not more than 0.5 ml of 80 percent acetone-water solution and later bring the final volume to 10.0 ml.

The chlorophyll content is determined by the measurement of light absorption, using a Beckman Spectrophotometer, Model DB, at a wavelength of 652 mμ. For best results the extract of chlorophyll

must not be stored for more than 12 hours even under darkness.

In all cases the contents must be secured to prevent evaporation and stored in a cool dark place.

APPENDIX II

STANDARD CURVE FOR CHLOROPHYLL DETERMINATION

The concentration (in mg/L) of chlorophyll in 80 percent by volume acetone-water solution can be determined from the equations developed by Arnon (4) and MacKinney (46) as shown below:

$$C_a = 12.7 \times D_{663} - 2.69 \times D_{645} \dots\dots\dots \text{Eq. A2-1}$$

$$C_b = 22.9 \times D_{645} - 4.68 \times D_{663} \dots\dots\dots \text{Eq. A2-2}$$

$$C_{a+b} = \frac{D_{652}}{0.0345} \dots\dots\dots \text{Eq. A2-3}$$

where D = optical density at respective wavelengths,
 C_a = chlorophyll a concentration in mg/L,
 C_b = chlorophyll b concentration in mg/L,
 C_{a+b} = concentration of chlorophylls a plus b.

To provide a rough check for the determination of total chlorophyll, the light absorption data for chlorophylls a and b were plotted by Arnon (4). The curves were found to intersect at the wavelength of 652 mμ. Hence a wavelength of 652 mμ is the optimum because various proportions of chlorophylls a and b least affect the results.

However, the wavelength and the sensitivity of an instrument should be checked before the coefficients in the above equations can be properly employed. The wavelength of the instrument used in this experiment, Beckman Spectrophotometer Model DB, was checked against the mercury lamp spectrum. The sensitivity of this instrument was checked by comparing the known quantity of chlorophyll with that

calculated from the above equations. The results indicated that the above equations could be suitably employed. Therefore, a standard curve for chlorophyll determination was prepared by plotting the straight line represented by Eq. A2-3.

APPENDIX III

APPARATUS AND METHOD FOR CONTINUOUS ALGAL CULTURE

The apparatus and method for the production of a continuous bacteria-free algal culture at a given density of population has been described by Myers (57). Because of its importance in providing a uniform experimentally reproducible algal source through the course of this research, a brief description is summarized here.

The use of algae or other microorganisms for physiological and biochemical investigations has the advantage of allowing the employment of large numbers of individual organisms and a resulting average-out of biological variation. Unfortunately different batches of microorganisms, obtained from different cultures or from the same culture at different times, are usually proved not to be uniform from one sample to another. For instance, in the case of the alga Chlorella, as a culture matures there occurs marked variations in the nitrate and hydrogen ion concentrations of the medium, in the conditions of carbon dioxide supply, and in the effective light intensity, or probably even in the auto-inhibitors etc. All of these internal variables are functions of the algal population or its growth phase.

If a culture is continuously diluted so as to be maintained always at one point on its growth curve, then effects of changing internal conditions may be eliminated entirely. This will then afford a means of producing a constant uniform algal culture. Following this concept an apparatus can be developed to maintain a constant algal culture at a given density of population by automatically diluting the growth

culture with fresh medium.

Figure A3-1 is a diagrammatic, cross-sectional view of the apparatus used in Laboratory of Algal Physiology, The University of Texas. The culture chamber is made of three concentric glass tubes providing an outer annulus J for circulation of constant temperature water and an inner annulus A containing the algal suspension. Outside dimensions of the chamber are approximately 6.0 cm diameter by 66.0 cm long; the thickness of the inner annulus A is about 6 mm, which gives a total content of about 400 ml of suspension.

The desired gas mixture is provided through the bubbler tube, B, which simultaneously provides carbon dioxide and agitates the algal suspension. The gas mixture may be first humidified by bubbling through a column of water held at the same temperature as the chamber.

Because of the requirement of rather rapid flow, 2 to 6 cc per second, the use of compressed air-carbon dioxide gas mixture has proved uneconomical. It has been necessary to devise a means of obtaining constant air-carbon dioxide mixtures. Outside air is delivered via a diaphragm pump at a constant pressure obtained by allowing the excess air to escape against a head of 8 feet of water. Carbon dioxide is delivered from a cylinder at a pressure of about four pounds per square inch by suitable mechanical reducing valves. The two gases pass through orifices so chosen that the resulting gas mixture has a composition of about 4.4 percent carbon dioxide. The actual rates of flow of the two gases are indicated by calibrated venturi flow gauges and occasional Haldane analyses have been made as over-all checks on the operation. The gas mixture is delivered at constant pressure by

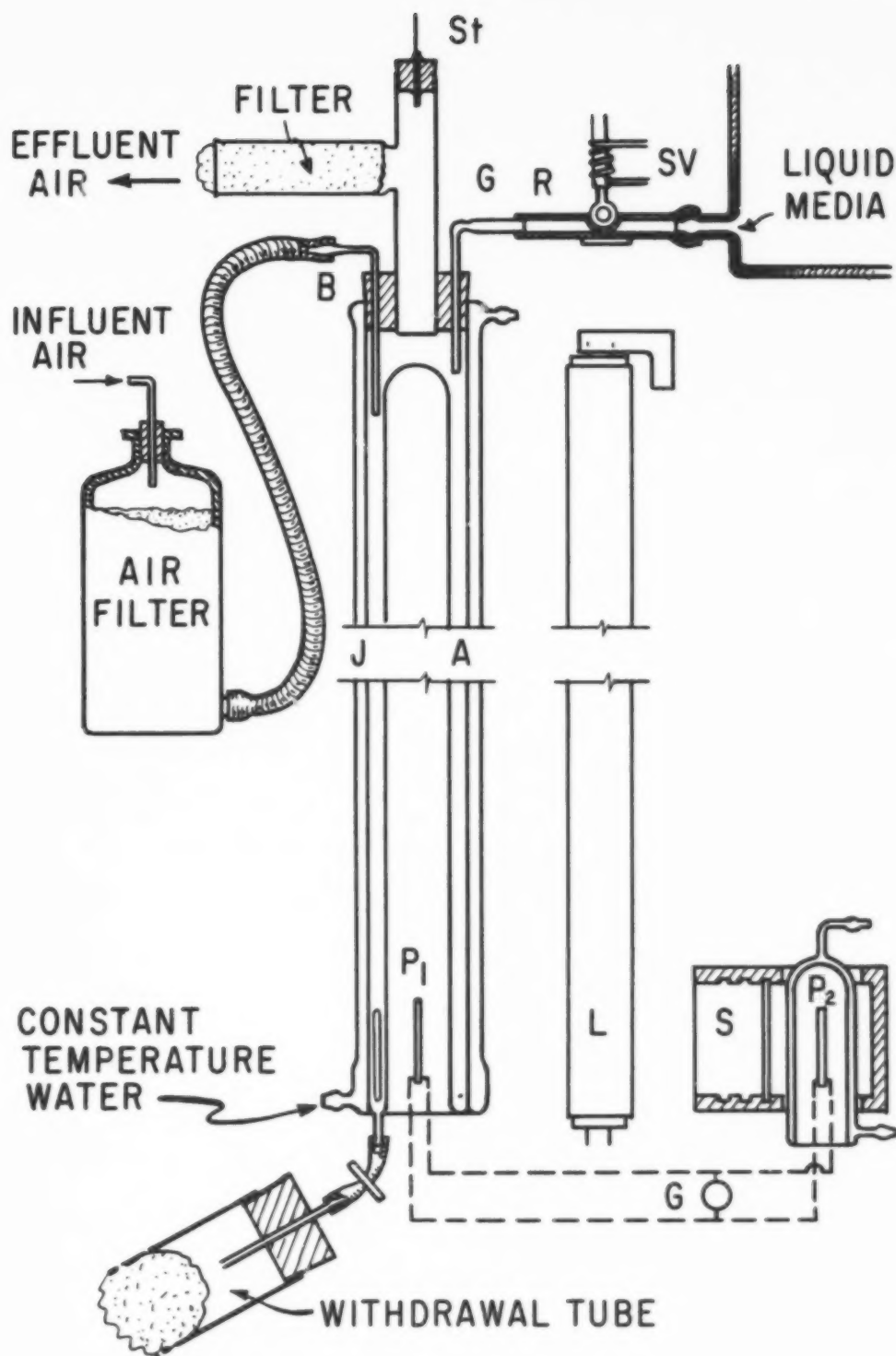


FIG. A3-1. CONTINUOUS CULTURE APPARATUS

allowing the excess gas to escape against a head of about 6 feet of water.

Sample of the suspension are harvested periodically by opening a screw clamp and allowing the suspension to run out through the withdrawal tube. Fresh medium from a large aspirator bottle is added through G by activation of a solenoid valve, SV, which lifts a plunger from rubber tube, R.

By suitable precautions pure culture conditions can be maintained. The withdrawal tube is protected by a glass skirt and cotton plug. After collection of a sample in a sterile flask a fresh cotton plug, previously autoclaved in a large test tube, is inserted in the glass skirt. Influent air is filtered through a 500 ml aspirator bottle packed with cotton. Effluent air passes out through a glass tube packed with cotton. The cotton filters are kept warm to insure against condensation of moisture. A light bulb placed underneath the the gas outlet adequately warms the effluent cotton plug. A heater for the influent filter is provided by a simple tin can and light bulb arrangement. Before setting up, the assembly consisting of the chamber, withdrawal tube, influent and effluent filters is sterilized by autoclaving. During this procedure a cotton plug is used in place of the rubber stopper, St, and the glass inlet tube for the medium is not connected to the aspirator bottle but is protected by a vial and cotton wrapping at G. The aspirator bottle is autoclaved separately, the end of the rubber tube, R, being protected by a vial and cotton wrapping.

After autoclaving, the chamber is placed in position, the clamp to the withdrawal tube closed, and a current of air passed

through. An inoculating suspension of cells from an agar slant is introduced through the uppermost opening and the cotton plug replaced by a sterile rubber stopper, St, fitted with a glass rod for ease in handling. The rubber tube, R, from the aspirator bottle is attached to the glass tube, G, with aseptic precautions.

The whole procedure of sterilization is quite cumbersome but it is required only at infrequent intervals of a month or more. New aspirator bottles of medium can be inserted by carefully removing the rubber tube of the old bottle, flaming the glass tube, G, and slipping on the sterile rubber connection of a new bottle. In operation a sample of about one ml is withdrawn daily into a flask of glucose-peptone broth as a check against bacterial or mold contamination.

Illumination is provided by four tubular fluorescent or tungsten filament lamps spaced symmetrically around the chamber. Here only one lamp, L, is shown in Fig. A3-1. Light intensity may be varied by changing the distance of the lamps from the chamber or by surrounding the lamps with sleeves made of wire screen.

The continuous culture apparatus becomes a steady-state device when constant cell concentration is maintained in the algal suspension. Fresh medium is added automatically by control of solenoid valve, SV, which is operated by a photometric device. Two photocells, P₁ and P₂, both of which are General Electric rectangular, barrier-type photocells $29/32$ inch x $1\frac{45}{64}$ inch, are arranged in a current-balancing circuit by connecting terminals of opposite polarity. The meter, G, which is Rubicon No. 3402, period 3.7 seconds and $R=335$ ohms, $CDRX=3900$ ohms, is a spot light galvanometer which has a No. 922 RCA phototube mounted

in place of the usual glass scale. The arrangement is such that an increase in light on the phototube energizes the relay. The relay operates the solenoid valve, SV.

The two photocells look at the same lamp. The inside photocell, P₁, is screened by the annulus of algal suspension while the outside photocell, P₂, is screened as desired by insertion of one of more uniformly exposed lantern slides, S. The outside photocell, P₂, is jacketed by a glass condenser through which is circulated the same constant temperature water used for the chamber; photocell and jacket are partially enclosed within a box arranged so that light enters only from the direction of the one light source. The inside photocell, P₁, is also screened with a piece of white paper so that its maximum output is about 25 microamperes. This decreases the sensitivity but greatly improve the stability of the photocell circuit.

In order to minimize ambient light effects on the photocells, the apparatus is partially enclosed in a box 30 x 30 x 36 inches high, painted white inside. The chamber just barely projects through a hole in the top of the box; and the air filters, solenoid valve, and aspirator bottle of medium are all mounted on top. The front of the box is a sheet of plywood with holes provided for ventilation and is easily removed when a sample is to be withdrawn.

In practice the original inoculum is allowed to grow until a desired population density is reached and the photocells are then balanced by inserting screens in front of the outside photocell. Thereafter a sample is withdrawn at a fixed time each day. The air flow is temporarily stopped and a small sample of approximately one ml is

collected in a flask of sterile broth as a check against contamination. The rest of the culture is then withdrawn down to a mark placed a centimeter or so above the top of the inside photocell. This leaves an inoculum of about 50 to 100 ml of suspension for the next day's growth. As the algae multiply illumination on the inside photocell, P_1 , is reduced. An off-balance current flows through the primary photocell circuit causing the galvanometer light spot to move across the phototube. The electronic relay then actuates the solenoid valve and allows new culture medium to flow in, diluting the algae and increasing the illumination on P_1 until a zero current again obtains through the galvanometer. In this way the algal suspension "grows" up the chamber. The design of the apparatus is such that conditions of illumination are independent of the total amount of the culture.

If sample are harvested from a culture at equal intervals, then the amount of the sample is an index of the rate of growth. It is also possible to harvest samples at any other desired time, provided that the culture is not allowed to run over. Daily samples of 200 ml to 300 ml containing 200 to 1000 cubic millimeter of packed algal cells are easily obtained. Satisfactory operation of the entire apparatus depends upon the stability and sensitivity of the primary photocell circuit. By maintaining the two photocells at constant temperature and keeping their illumination at a low level good stability may be obtained over a period of weeks. The over-all sensitivity is such that the solenoid never allows more than one percent of the volume of the culture to flow in during one relay cycle.

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